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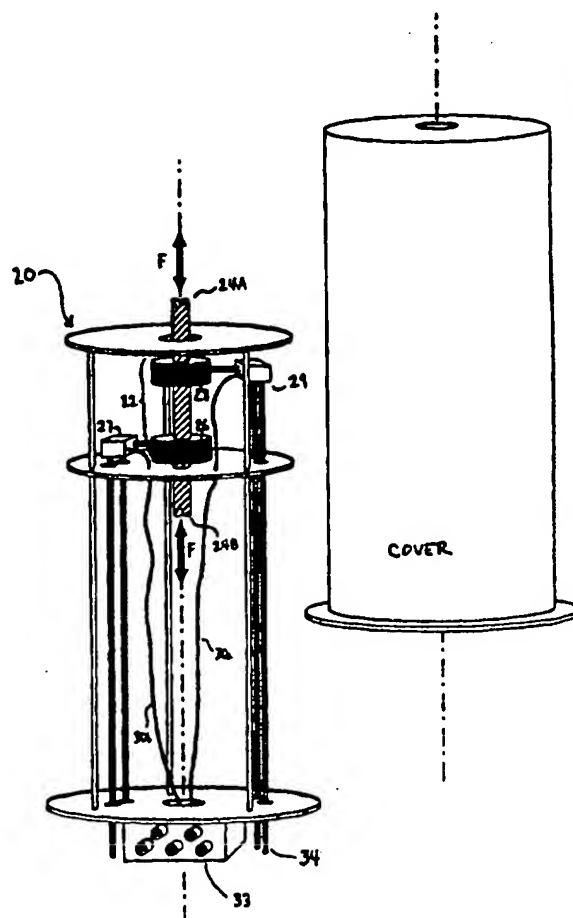
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(54) Title: TANDEM COIL NMR PROBE

## (57) Abstract

An NMR probe (20) positions a flow chamber (22) with first and second flow regions in the high field of an NMR apparatus. A second, downstream, flow region is surrounded by an exciter/detector coil which may be of a conventional type for home- or heteronuclear detection, while an upstream, first region is excited by an antenna to condition or enhance a downstream measurement. The downstream coil (26) is tuned to detect heteronuclear resonances, while the upstream coil may be tuned for enhancement of the same or a different species. A cavity, in conjunction with the upstream coil (28a), allows populations and transfer coherence excitation between electrons and nuclei.



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## Tandem Coil NMR Probe

### *Background of the Invention*

Nuclear magnetic resonance (NMR) is a useful technique for determining molecular structure. Nuclei which have an odd atomic number or odd atomic mass possess magnetic moments and angular momenta. Such nuclei, when placed in an applied external magnetic field, will precess about the field with a frequency which depends upon the strength of the applied field  $B_0$  and the gyromagnetic ratio  $\gamma$  of the nucleus. Since every distinct nucleus has a unique gyromagnetic ratio, the precession frequency for each is different in a given magnetic field. For commonly used nuclei and conventional magnetic field strengths, the precession frequency is in the radio frequency (RF) range. If RF energy is applied at the appropriate frequency, the precessing nuclei will be excited and will consequently generate a signal which can be detected and processed to yield an NMR spectrum. These spectra contain a wealth of structural information and have made NMR spectroscopy one of the most versatile means for determination of the structure and conformation of molecules.

NMR is, however, a relatively insensitive spectroscopic technique, and typically requires large amounts of sample, compared to other spectroscopic methods, to yield useful results. This lack of sensitivity has restricted the use of NMR to those cases where considerable sample is available. Although it has been known for many years that NMR signals are enhanced in flowing liquids, the large sample volumes required to create a flow generally offer no benefits for fields such as protein analysis where the small quantities of sample available for analysis would require so much dilution as to negate any advantage gained by flow NMR.

There have been several reported applications of flow NMR in recent years. These have included liquid chromatographic detection (E. Bayer et al. *J. Chromatogr.*, **186**, 497 (1979); F. Haw-James et al., *Anal. Chem.*, **52**, 1135 (1980)), measurement of flow rates and concentrations (A.I. Zhernovoi and G. D. Latyshev, "Nuclear Magnetic Resonance in a Flowing Liquid", Consultants Bureau, New York, (1965); M.A. Hemminga and P.A. De-Jager, *J. Magn. Reson.*, **37**, 1 (1980); H.S. Lew, U.S. Patent 4,901,018), detection of short-lived reaction intermediates (C.A. Fyfe, M. Cocivera, and S.W.H. Damji, *Acc. Chem. Res.*, **11**, 277 (1978)), in vivo organ perfusion studies (N. Lavanchy et al. *Card. Adapt. Hemodyn. Overload, Train. Stress, Int. Erwin Riesch Symp.*, 228 (1983); K. Albert et al., *Z. Naturforsch. C: Biosci.* **39C**, 859 (1984)), and dynamic electron-nuclear polarization (H.C. Dorn et al., *J. Magn. Reson.*, **79**, 404 (1988); R. Gitti et al, *J. Am. Chem. Soc.* **110**, 2294 (1988); S. Stevenson and H.C. Dorn, *Anal. Chem.*, **66**, 2993 (1994)). Still, many potential uses of flow NMR have not yet been made practical.

A further disadvantage of previously reported flow NMR systems has been the inability to exploit the many applications of NMR spectroscopy which make use of nuclear Overhauser enhancement or heteronuclear polarization transfer to enhance or otherwise clarify certain spectra. These phenomena have supported impressive advances in sensitivity and range for the analysis of non-flowing samples, but are not well adapted to flow NMR applications, because of difficulties associated with the evolving nature of these states and the isolated structures of NMR detection apparatus. These factors have generally limited implementation of nuclear spin enhancements to arcane experimental configurations of equipment, or to the measurement of static samples in conventional equipment.

Accordingly, it would be desirable to provide apparatus capable of implementing nuclear spin enhancements and performing observations with an increased range or greater sensitivity.

It would also be desirable to perform enhanced observations with heteronuclei, in flowing samples, or preferably both.

All of the above-cited references and publications, as well as the further publications specifically identified in the disclosure below are hereby incorporated herein by reference in their entirety for details of NMR machine construction and measurement techniques.

### *Summary of the Invention*

The present invention provides a novel probe for use in flow NMR spectroscopy. In one aspect of the invention, the flow probe includes a flow vessel which is positioned by a housing in a high magnetic field, and with tandem transmitter/receiver coils positioned sequentially along a fluid flow path through the vessel. This arrangement permits the use of nuclear Overhauser enhancement, and enhancement by coherence transfer techniques, such as spin-temperature labelling, of resonant signals by excitation of the sample nuclei in the flow volume at the upstream coil and detection at the flow volume of the downstream coil, while controlling difficult experimental factors such as residence time in the field.

In certain embodiments of the present invention, one or both of the upstream or downstream flow volumes includes a microwave cavity and a source of immobilized free radicals (IFR), which in this instance are configured to couple electron spin polarization to nuclear species, while retaining the closely-spaced high field NMR geometry and its functional advantages. The probe apparatus allows unique experimental protocols, for example, simultaneous excitation of a selected nucleus of the sample and also the unpaired electrons of the IFR material. This allows the implementation of electron-nuclear cross-polarization enhancement (also referred to as dynamic nuclear polarization or "DNP") of the

sample resonances, with potentially large increases in detection sensitivity of the sample resonances at the downstream coil.

5 In other embodiments of the invention, the flow path may include a reaction chamber situated so as to allow the initiation of a reaction and detection of the intermediates or products of that reaction at the downstream coil. With an upstream RF coil, certain of the reactants may be labeled or otherwise conditioned before reaction and detection.

10 In still other embodiments of the invention, a recirculation path, preferably with an integral pump, is provided to recycle the flowing fluid from a downstream point to a point upstream forming a closed loop flow path. In this fashion, the sample may be recirculated, allowing quite small volumes of sample and solvent to be used even for lengthy measurements taking hours or days.

15 In yet further embodiments of the invention, the probe may include a means for varying the size of a central part of the flow path to selectively control the flow time between the upstream and downstream coils. Such variation of the flow time adds an additional dimension in a multidimensional experiment such as reaction monitoring or the like.

20 In still further embodiments, the probe may be configured with two or more coils which are each tuned to separate nuclear Larmor frequencies with separate impedance matching and tuning means, for fine-tuning the resonance frequency and impedance of one or more of the coils, as may be necessary from one sample to the next. One or more of the coils, preferably the downstream coils, can be doubly-tuned, with one frequency corresponding to the Larmor frequency of deuterium or another suitable lock signal.

Novel protocols utilizing the probes include multidimensional and spin-temperature labeling procedures.

### 25 *Brief Description of the Drawings*

Figure 1 depicts a generalized NMR apparatus.

Figure 2 shows an NMR probe according to the present invention.

Figure 3 illustrates RF coils disposed around a flow vessel in the subject NMR probe.

30 Figure 4 is a schematic representation of one embodiment of the subject NMR probe disposed in a magnet.

Figure 4A shows another embodiment similar to that of Figure 4.

Figures 5 shows an embodiment of the subject probe having a microwave cavity.

Figure 6 shows an embodiment of the subject probe with a reaction chamber and flow delay.

35 Figure 7 shows a  $^1\text{H}$  spectrum detected by a probe embodiment with toroidal coil.

Figure 8 shows detection sensitivity of the probe as a function of flow rate and flip angle.

Figure 9 shows  $^{13}\text{C}$  spectra of ethanol detected by the probe.

Figure 10 shows  $^{13}\text{C}$  spectra of  $\alpha$ -pinene detected by the probe.

Figure 11 shows another probe with multiple stages.

Figure 12 illustrates one embodiment of a flow delay element for use in probes of the  
5 invention.

Figures 13A and 13B illustrate another flow delay element.

Figure 14 illustrates a pulse sequence for a two-dimensional NMR measurement procedure.

Figure 15 illustrates a  $^1\text{H}$ -decoupled  $^{15}\text{N}$  spectrum of 80% N-methyl formamide in  $\text{CDCl}_3$ .

Sweep width 1850Hz. Spectrum from bottom to top: Static spectra; flow rate of 6.0 mL/min  
10 with CPD decoupling, toroid inactive; flow rate of 6.0 mL/min with CPD on toroidal cell  
during recycle delay; DEPT spectrum under optimized conditions.

### *Detailed description of the Invention*

The uses of flow NMR reported in recent years have typically been restricted to those  
15 applications where large amounts of sample are available. Traditional means of enhancing  
sensitivity in static NMR experiments, such as nuclear Overhauser enhancement (NOE) or  
polarization transfer methods, have not been applicable to flow NMR studies. The ability to  
extend such techniques to flow NMR would be a valuable contribution toward increasing the  
versatility of flow NMR. The present invention addresses this problem by a novel NMR  
20 probe for use with conventional NMR spectrometers and magnets to perform NMR  
measurements on flowing fluids.

The term "flow NMR" as used herein refers to NMR spectroscopy of flowing fluids.  
Fluids contemplated for use in the invention include liquids, gases, supercritical fluids, and  
other fluids which are compatible with the probes of the present invention.

25 The lack of sensitivity of NMR techniques has been a severe hindrance to use of  
NMR methods, particularly in cases where the sample spectrum is complex and data  
acquisition times are long. The importance of increasing sensitivity can hardly be overstated.  
In modern research, the amount of sample available for spectroscopic measurement is  
frequently very small, and the difficulty of performing NMR analysis on minute quantities of  
30 sample often results in long data acquisition times if only small amounts are available, or  
else delays until more sample can be obtained. If the sample is unstable, long analysis times  
are simply not possible. The probe of the present invention increases sensitivity and  
decreases the time needed to accumulate sufficient data. The subject probe thereby makes the  
study of unstable compounds more facile. At many institutions, the amount of NMR  
35 instrument time available is limited and is carefully rationed; increased sensitivity helps to  
allocate this important resource more broadly. The probe also makes possible the use of  
lower concentrations of sample as well as smaller quantities of sample. The concentration of

the sample may be of crucial importance. For certain samples, for instance molecules which are capable of self-aggregation, changes in concentration may result in changes in the NMR spectrum, so that they can be detected, if at all, only when extremely dilute. Reaction products or intermediates may be both short-lived and present in minuscule quantities, and hence may be unobservable by NMR unless the sensitivity can be enhanced by an order of magnitude or more. The probe of the present invention therefore makes it possible to perform NMR measurements over a range of conditions which are not amenable to conventional systems.

Figure 1 shows the general features of an NMR apparatus which has a large electromagnet 1 generally maintained in a superconducting state within a housing filled with liquid helium and insulated by an outer shell filled with liquid nitrogen, a probe 2 which fits centrally within the housing to position a sample in a region of high and uniform magnetic fields, and a control and measurement system 3 which provides the necessary drive signals and receives the NMR signals for irradiation, detection and processing to derive a spectrum from a sample inserted in the apparatus and held by the probe. A conventional probe includes a vessel which holds the sample, an RF coil which surrounds the sample, and various tuning and impedance matching elements; generally speaking, trimmer capacitances and inductors which are connected to non-magnetic rods which serve as control elements for performing fine adjustments of the coil. These elements all connect to input-output terminals at the base of the probe, and the probe thus adapts the samples to reside in the inaccessible cryogenic center of the magnet and enables the necessary controls and measurement signals to be taken without interfering with the magnet.

Various details of construction and processing have become commonplace in NMR apparatus, and will be assumed without further discussion to be included in embodiments of the invention described below. Thus, the coils used in the subject probe may optionally be double tuned to the resonant frequencies of two different nuclear species, and the RF receiver coils in the subject probe are preferably configured to allow quadrature detection, or quadrature processing of detected fields. Furthermore, the probe of the present invention may be used with any magnet capable of providing a strong magnetic field, including permanent (iron) magnets, electromagnets, and superconducting magnets. When reference is made to a highly uniform field, it is understood that such uniformity may be achieved by providing a set of "shim" coils, which are energized to supplement and correct the basic static field. This is generally done under computer control in response to detection of the resonance frequency and linewidth of a simple species.

Figure 2 illustrates a basic embodiment of the probe 20 according to the present invention. As shown, probe 20 includes a vessel section 22 to be positioned in the magnet field, connectors 24a, 24b for placing the vessel in a flow segment, and coils 26 and 28

placed consecutively along the flow path through the vessel. Arrows F indicate the flow axis. In the discussion below, the terms "upstream" and "downstream" will be used without reference to whether the flow is actually up or down in the illustration of Figure 2: the actual flow direction may be selected depending on considerations of filling and bleeding of the vessel and other such factors. However, we shall routinely use the terms "upstream" and "downstream," generally identifying the downstream coil 26, illustrated in Figure 2, as the "primary" coil, meaning the one which performs a principal or ultimately intended nuclear magnetic resonance measurement, unless otherwise indicated or clear from the context. Thus, in discussions of measurements taken upon species flowing through the vessel, the RF signal induced in coil 26 will be subject to Fourier transform analysis, and the frequency spectra of the precessing nuclei will be generated from the signals therein.

Continuing with the description of Figure 2, each coil 26, 28 has associated therewith circuitry 27 and 29, respectively, for performing fine adjustments to the tuned frequency and impedance. Each coil is connected by leads, of which two, 30a and 30b, are shown, to shielded input-output junctions 33 in the base of the housing. In practice, the circuitry 27, 29 are located above or below the actual coil regions, so as not to affect field uniformity, but are illustrated adjacent to the flow region for expository clarity. Input-output junctions are set up to match an RF signal cable of standard impedance characteristics. In addition, a series of adjustment/control rods 34 of a non-magnetic material extend from outside the probe housing to the circuitry 27, 29 for performing fine adjustments of the capacitors or inductors and the coils. With this arrangement, the probe is set up to apply to and to receive RF signals from nuclei in two distinct regions along the flow path. The advantages of this construction will be discussed below in various examples. A more detailed description of the coils and flow vessel will be given with respect to Figure 3.

In Figure 3, the coils 26, 28 are shown disposed sequentially about the flow vessel 22. The coils are shown as saddle coils, but it will be understood by one skilled in the art that other known coil configurations for applying RF fields may be used. The flow vessel 22 extends through a conditioning zone 40, a transition zone 45 and a primary detection zone 50. In the present invention, various forms of conditioning or measurement are contemplated in zone 40 upstream of the primary coil as well as, in certain embodiments, a reentrant flow path for effecting enhanced measurements.

Figure 4 shows the probe of Figure 3 disposed within the magnet. A pump 64 (such as a pneumatically driven pump) and a recirculation loop 62, both shown in phantom, are preferably provided when analyzing small amounts of sample. The RF coils 26 and 28 are transmitter/receiver coils, and each coil preferably further includes an additional, generally one or more concentric coil (not shown in the figure) surrounding the same flow volume, as is known in the art, for providing a field/frequency lock (e.g. a deuterium lock) or a nuclear



magnetic resonant frequency different from that of the respective accompanying coil, as for example in decoupling and heteronuclear correlation experiments. In certain preferred embodiments, one or more of the coils can be tuned to more than one frequency, e.g., such as tuned to both  $^1\text{H}$  and  $^2\text{D}$ . Likewise, it will be understood that the pump mechanism can be  
5 (optionally) partially disposed or completely disposed outside of the probe housing, particularly where it is desirable to use an induction motor or the like.

The terms "coil" and "RF coil" are recognized in the art and are used interchangeably herein. The term "RF coil" refers to an electrical conductor, e.g., wire or tape with non-zero inductance, which is often coiled shaped and which can be used to transmit and receive  
10 radiofrequency signals. RF coils are preferably made of copper or other electrically conductive but non-ferromagnetic materials. The RF field generated by a coil is preferably orthogonal to the strong applied magnetic field  $B_0$ . Exemplary RF coil configurations which may be useful in the present invention are solenoidal, saddle, Helmholtz, modified Helmholtz, Golay, and toroidal coils. Preferred embodiments employ coils providing  
15 uniform fields.

In the discussion which follows, the invention will be applied to difficult measurement problems which have arisen in modern NMR equipment having very high magnetic fields. In general the degree of resolution and sensitivity obtainable depend on the field strength as well as on the homogeneity achievable in the field. Typical high-  
20 performance machines are characterized by proton resonant frequencies of 400 MHz to 750 MHz, corresponding to magnetic fields of 9.4 to about 18 Tesla; however, the principles of the present invention are not limited to such machines. The terms "high field" and "high magnetic field" as used herein are intended to refer to a magnetic field with a field strength of about at least 0.5 Tesla (T), preferably in the range of about at least 1T to about 24T, and  
25 more preferably about at least 2.1T to about 24T.

### Example 1 (Prototype Embodiment)

Figure 4A schematically shows the design of a sequential-coil flow-probe in one prototype embodiment. The  $^{13}\text{C}$  transmit/receive downstream coil 26 surrounded a 5 mm o.d. Pyrex vessel with a volume of 0.19 ml and was surrounded by a  $^1\text{H}$  decoupling coil (not  
30 shown) of conventional type. The Pyrex cell was joined to Teflon tubing with Teflon tape.

A prepolarization chamber which is large compared to the detection coil helps the sample attain Boltzmann equilibrium and NOE enhancement before detection. Two types of prepolarization chambers have been constructed, giving comparable enhancements. One was  
35 built of 10mm Pyrex tubing inside a conventional saddle-shaped Helmholtz  $^1\text{H}$  coil. The other, depicted as a toroidal coil 28a was used as the upstream coil to enclose a large volume.

with a geometry that allowed it to be mounted close to the homogenous region of the magnet, and has a large filling factor with good Q (T.E. Glass and H.C. Dorn, *J. Magn. Reson.* **51**, 527 (1983)). The toroid 28a was made by winding six turns of commercially available coil wire (silver-plated copper) around a Teflon spool. The sense of winding was reversed after  
5 the first three turns to reduce the inductance of the coil and facilitate tuning to high frequencies (S.B.W. Roeder and E. Fukushima, *J. Magn. Reson.* **59**, 307 (1984)). The toroidal coil 28a was insulated with Teflon tape to prevent arcing. Around the spool inside the toroid, approximately 1.3 m of Teflon tubing (1/16 inch inner diameter) was wound up, holding 1.6 ml of sample. This design avoids excessive sample mixing during the passage  
10 through the toroid. An alternative design employing a double-saddle Helmholtz coil around a 10 mm diameter Pyrex cell has also been tested, and provided comparable results.

The tuning circuit for the upstream coil 28a, including three non-magnetic high voltage Johanson variable piston capacitors (0.8 - 10pF), was mounted above the transmit/receive coil of a commercially available BRUKER <sup>13</sup>C probe which was suitably  
15 modified to receive the additional coil and tuning circuitry, and good grounding was achieved by a solid connection to the housing of the probe. Connection to the circuit was made with a non-magnetic 50 ohm coaxial cable lowered through the magnet bore and fastened to a Sealelectro SMB jack on top of the probe. The center of the toroid coil 28a lies 3.2 cm above the primary transmit/receive coil 26. Figure 7 shows a <sup>1</sup>H "spectrum" recorded with the  
20 toroidal coil. The signal has a width of almost 20 KHz and is shifted by almost 20 KHz, relative to that obtained at the decoupler coil of the primary coil 26 below. The sharp signals visible in the center of the spectrum come from the decoupler coil which resonates with the toroid.

The low power output of the proton decoupler of a BRUKER AM 400 NMR  
25 instrument was further amplified by a 2W linear amplifier to apply a composite pulse or continuous wave (CW) RF signal to the toroidal coil. Series crossed diodes and a 400MHz bandpass filter on the amplifier output were employed for reducing noise in both observe and deuterium lock channels. The 90° flip angle of the toroid was approximately 140 ms.

A Millipore peristaltic pump employing 5mm o.d. Masterflex Tygon tubing was used  
30 for sample recirculation.

#### Determination of Optimal Parameters

To show the individual contributions of flow and NOE enhancement, the acquisition parameters for each experiment were optimized within the hardware limitations discussed above. Static experiments were optimized according to well-known principles (R.R. Ernst et al., *Principles of Magnetic Resonance in One and Two Dimensions*, Clarendon Press, Oxford,  
35 1987). The acquisition time  $t_{aq}$  is determined by the necessary sweep width and digital

resolution (in practice, acquiring for 2 to 3 times  $T_2^*$ , the effective transverse-relaxation time). Keeping pulse repetition time  $T_{rep}$  less than  $T_1$ , choosing pulse angle  $\beta$  equal to the "Ernst angle" (Ernst et al., *supra*), and applying matched filters before processing leads to optimal  $(S/N)_1$ . In flow experiments,  $T_{rep}$  must be suited to the flow rate so that  $T_{rep}$  equals  $\tau_{det}$ , the sample lifetime in the detection cell ( $\tau_{det} = \text{volume}_{det}/\text{flow rate}$ ). Our simulations of steady-state magnetization are based on a classical vector model with plug flow enable determination of relative sensitivities, optimal flow rates, pulse angles  $\beta$ , and  $T_{rep}$ . Figure 8 calculated for parameters typical for ethanol shows that  $(S/N)_1$  reaches a maximum for a specific flow rate and pulse angle. For  $T_1$  and  $T_2^*$  values of 3.5 and 0.1 s, and a  $t_{aq}$  of 0.25 s, our simulations reveal optimal  $(S/N)_1$  for a flow rate of 19.3 ml/min, a pulse angle of  $90^\circ$  and a  $T_{rep}$  of 0.59 s. Our studies show that the correct flip angle for flow NMR is always  $90^\circ$  when  $T_{rep}$  equals  $\tau_{det}$ , and the flow rate is optimal when  $\tau_{pre}$ , the sample lifetime in the prepolarization volume, is between 1.1 and 1.8  $T_1$ .

### Results and Discussion

Some proton-coupled  $^{13}\text{C}$  spectra of 90 % ethanol in  $\text{D}_2\text{O}$  containing a small amount of acetic acid (for catalyzing proton exchange) are depicted in Figures 9A-9D. To compare sensitivities of spectra, we determined optimal parameters for each individual spectrum, and used always the same total acquisition time. For a static sample (zero flow rate), the best response is achieved employing a flip angle of  $\beta = \arccos(e^{-T_{rep}/T_1})$  (R.R. Ernst and W.A. Anderson, *Rev. Sci. Instrum.* **37**, 93 (1966)). The longitudinal relaxation times of the methyl and the methylene group of ethanol are 5.5 s and 3.5 s, respectively. The spectra recorded with a repetition time of 2.2 s and a flip angle of  $60^\circ$  are shown in Figure 9A. All spectra have been processed using a matched filter (exponential line broadening, 3Hz).

To obtain full premagnetization and optimal flow signal enhancement, a flow rate of 25 ml/min was chosen. The optimal repetition time for a 0.19 ml detection cell is 0.45 s. To obtain a satisfactory digital resolution, 4K of data points were recorded at a sweep width of 8065 Hz. This set of parameters leads to an acquisition time of  $t_{aq} = 0.25$  s. Twelve scans were coadded, preceded (as in all spectra reported here) by eight dummy scans). For these parameters, the duty cycle of the toroidal coil 28 is only 44%, because the composite pulse decoupling (CPD) sequence cannot be applied to the toroid during acquisition. This is a consequence of the fact that the decoupler coil resonates with the toroid 28a, which causes partial decoupling during acquisition. A circuit that untunes the decoupler coil during acquisition would most likely remove this problem, and this would be provided in a further embodiment by a synchronous switching circuit that connects an additional inductive or capacitive circuit element to the decoupler coil to shift its resonance and damp oscillations during acquisition. For the present experiments, a compromise that provided a better duty cycle (89%) for the toroidal coil 28a was chosen with a flow rate of 6 ml/min and a repetition

time of 2.2 s. This set of parameters allows the sample to experience only one 90° pulse in the detection cell; however the prepolarization and NOE buildup are both reduced. Figure 9B shows the spectrum at a flow rate of 6 ml/min without any RF pulses applied to the toroid 28a, in which it is seen that the S/N ratio is approximately twice that of the static spectrum.

5 The NOE enhanced spectrum induced by a CPD sequence applied through the toroid 28a (gated off during acquisition) is shown in Figure 9C. Despite the inhomogeneity of the magnetic field around the toroid and the non-optimal flow rate, a NOE enhancement factor of 2.3 is achieved. DEPT (Distortionless Enhancement of Polarization Transfer) spectra (Figure 9D) give an even higher enhancement; however it suffers from the fact that the signals of

10 quarternary carbons completely disappear from the spectrum. The DEPT spectrum has been recorded with a shorter repetition time and an increased number of scans (16 scans, but the same total acquisition time), in view of its dependence on the relatively shorter relaxation times of the protons.

Some <sup>13</sup>C spectra of α-(+)-pinene (40% in CDCl<sub>3</sub>) are shown in Figure 10. The α-

15 pinene has been distilled prior to the acquisition of the spectra. <sup>13</sup>C longitudinal relaxation times at 100.26 MHz ranged from 17 s (C6), 14 s (C2), 8.5 s (C1), 7 s (C3, C5) to ~5s (C4, C7, C8, C9, C10). Acquisition conditions were optimized for the longest T<sub>1</sub> and were as follows. Spectrum 10A was recorded with no flow, repetition time 3.2 sec., 48 scans CPD decoupling during acquisition with the toroidal coil 28 inactive. Spectrum 10B was recorded

20 with a flow rate of 1.9 ml/min., repetition time of 3.2 seconds, CPD or CW on the toroidal coil during recycle delay, and CPD decoupling on the downstream decoupler coil during acquisition with the toroid inactive. Spectrum 10C was taken under the optimized conditions, at a flow rate of 1.9 ml/min, repetition time of 1.8 sec., 64 scans. The flow rate was adjusted by trial and error, and showed that a flow rate of 1.9 ml/min gave a much better response than

25 the calculated flow rate of 4.5 ml/min. This is a consequence of the higher viscosity of α-pinene, which causes a pronounced laminar flow effect. Thus, part of the sample passes the prepolarization coil before the full magnetization is built up. This cannot be compensated by another fraction of the sample that stays longer than required in the prepolarization volume, because the maximum longitudinal magnetization is limited by the strength of the magnetic

30 field. However, the higher T<sub>1</sub> values can in principle be offset by a larger pre-polarization volume.

All pinene spectra are recorded with the same overall acquisition time of 115.4 s. Composite pulse decoupling was achieved by switching between the low power decoupler for the toroid and the high power decoupler for the decoupler coil, whose carrier frequencies

35 differed by 20 KHz. In initial experiments, simultaneous excitation of both coils was not possible, because only one decoupler was available. A higher number of data points (16 K) and, consequently, a longer acquisition time, allowed a duty cycle of approximately 80% for the toroidal coil to be achieved.

The overall enhancement factors obtained for  $\alpha$ -pinene are between 1.5 and 2 for a flow rate of 1.9 ml/s using the toroidal coil for NOE enhancement, and are very similar for flow DEPT. The quarternary carbon signals (C2, C6) completely disappear in the DEPT spectrum (Figure 10C).

5  $^1\text{H}$ -decoupled  $^{15}\text{N}$  spectra of 80 vol % *N*-methylformamide ( $T_1 = 11.8$  s) in  $\text{CDCl}_3$  depicted in Figure 15 were recorded with the same probe with the  $^{13}\text{C}$  coil retuned to  $^{15}\text{N}$ . The resonances of all spectra in Figure 15 are phase inverted because of the negative NOE of  $^{15}\text{N}$ , even for a  $^1\text{H}$ -decoupler duty cycle ( $T_{\text{rep}}/t_{\text{aq}}$ ) as low as 8% in the static spectrum (Figure 15, bottom spectra). The flowing of sample in Figure 15 results in reduced NOE because of  
10 lower  $\tau_{\text{det}}$ , which tends to cancel the signal despite the increased decoupler duty cycle (32%). Strong NOE enhancement is shown in Figure 15 (2nd spectra from top) with the decoupler time-shared between the toroidal and decoupler coils (toroidal duty cycle = 68%). Our simulations for this case predict an overall enhancement (flow + NOE) of -8.8. The  $^{15}\text{N}$  DEPT spectrum (see Figure 15, top spectra) with a  $T_{\text{rep}}$  reduced to accommodate the smaller  
15  $T_1$  of the directly bonded amide proton, gave approximately 30% higher  $(S/N)_1$  than the spectrum recorded with CPD on toroidal cell during recycle delay.

It should not be very surprising that, given enough time, the NOE can be obtained for flowing samples, or that enhancement can be accomplished with relatively small amounts of RF power. The amounts of NOE enhancement are orders of magnitude less than that  
20 achieved in flow DNP (S. Stevenson and H.C. Dorn, *Anal. Chem.* 66: 2293(1994)), but for many applications, e.g., metabolic studies and LC detection, it should be well worth the modest addition in hardware to recover this normally wasted sensitivity.

What is perhaps more significant is a host of new possibilities suggested by the tandem flow-cell arrangement for analytical FT NMR, including the study of reaction  
25 mechanisms, short-lived intermediates, and pathways by "spin-temperature labeling." For example, a reactant resonance in an upstream cell could be inverted by a selective pulse prior to rapid mixing with a chemical reagent and detection in a cell downstream. This could provide an alternative to expensive and time-consuming isotopic labeling, for example, in the study of molecular rearrangements. Such applications would require magnetic homogeneity  
30 at the upstream cell comparable to that existing downstream, possibly demanding a special magnet and/or dual shim systems. The NOE enhancement method describe here, however, requires little magnetic homogeneity upstream and should be applicable to all heteronuclei where  $^1\text{H}$  dipolar interactions contribute to relaxation.

Returning now to a description of the apparatus, the invention also contemplates a  
35 probe including a flow chamber having an NMR detection region, and further including a microwave cavity located along the flow path for polarization conditioning or signal enhancement of a nuclear type of interest. One such device is shown in Figure 5, wherein a

microwave cavity 52 is provided at the upstream coil 28, and an environment of immobilized free radicals 54 in the flow region 40. Thus the microwave cavity 52 is situated in the magnetic field, and the material passing along the flow path is simultaneously subjected to the stationary magnetic field  $B_0$  and to interaction with the captive electrons of the radicals  
5 immobilized in the microwave cavity, as well as the RF fields  $B_1$  from the upstream coil. A representative example of operation of this probe follows.

### Example 2

In an exemplary protocol, a sample is introduced at an upstream flow position and  
10 flows into the microwave cavity 52 to interact with a source of immobilized free radicals (IFR) on a solid support. Suitable sources of IFR are known, and may be, for example, silica-phase immobilized nitroxide radicals (R. Gitti et al., *J. Am. Chem. Soc.* 110, 2294 (1988)). Other free radicals such as phenoxide have been reported, and the choice of species  
15 may depend in part on considerations of compatibility with the sample as well as empirical selection factors such as efficiency and ease of preparation. The microwave cavity is a conductive walled chamber which resonates at the microwave frequency, discussed further below.

Briefly, the following protocol seeks to enhance the polarization (hence detectable signal) of a with a small gyromagnetic ration nucleus by introducing the desired spin into an  
20 abundant electron species and coupling that spin into the nucleus. The technique used is analogous to the classical technique of cross-polarization-magic angle spinning (CP-MAS) used to couple spin polarization between pairs of nuclei in solids, to take advantage of the greater excited state population available in one species. This polarization coupling requires that the target nucleus be conditioned to change spin upon receiving the quantum of energy  
25 available from a spin transition in the abundant donor nucleus, generally a proton. Because the electron gyromagnetic ratio is much greater than that of e.g. carbon ( $\gamma_e/\gamma_C = 2628$ ), and its precession frequency correspondingly great, conventional wisdom would not normally suggest NMR signal enhancement by electron-nuclear spin interactions. This technique is known in the art as Dynamic Nuclear Polarization.

The probe of Figure 5 provides necessary elements for controlling such a coupling  
30 interaction between electrons and nuclei. As described further below, this is done using the upstream cavity and coil by spin-locking both the electrons and the nuclei of interest away from the static magnetic field  $B_0$  and arranging that the strength of the orthogonal magnetic fields induced by the two irradiation sources satisfies the Hartman-Hahn condition on  
35 gyromagnetic ratios for spin coupling of two particles.

The nuclear type of interest is excited by spin-locking according to established methods (for details of suitable spin-locking procedures, see e.g. G.A. Morris and R. Freeman, *J. Am. Chem. Soc.* **101**, 760 (1979)), so that the spin is locked in the magnetic field  $B_1$  of the RF coil, which in this case is the X-Y plane, orthogonal to  $B_0$ . In an analogous fashion, the microwave transmitter establishes a standing microwave field with a magnetic component rotating in the X-Y plane, which interacts with the spin of the unpaired electrons of the immobilized free radical phase so that these electrons are also spin-locked and in the desired orientation. As with the corresponding nuclear-nuclear cross-polarization coupling techniques, the two fields are preferably locked in phase relation. For lower static magnetic fields  $B_0$  (hence longer microwave electron spin resonance wavelengths), this may be accomplished with a doubly resonant cavity/coil configuration. For higher microwave frequencies necessary in very strong static magnetic fields, this may be achieved by suitable frequency dividing and phase feedback controls, possibly with special mixing or doubling resonators operative in the short microwave region. As with nuclear cross-polarization, the amplitudes of the two locked fields are adjusted to satisfy the Hartman-Hahn ratio. Preferably, the electrons are spin-locked before the nuclei, though electron spin-locking can be accomplished simultaneously or after spin-locking the nuclei, and the RF field then applied to the nuclei to bring both spin systems into alignment. The polarization of the excited free radicals is then transferred to the spin-locked nucleus of interest as the fluid flows over the solid support. Since electrons have a much greater energy difference between up and down spins, this leads to a greatly enhanced population of the coupled spin polarization in the flowing nuclei. When the fluid flows downstream, this increased polarization is detected at the second coil as a greatly enhanced signal in the spectrum of the nuclei. The construction of the upstream coil in combination with the microwave cavity and immobilized free radicals thus provides a structure for exciting electron spin and coupling this spin to a heteronucleus. This mechanism may in theory produce signal enhancements of up to 2628 for  $^{13}\text{C}$  and 6570 for  $^{15}\text{N}$ . Such enhancements would correspond to reductions in data acquisition time for a two hour experiment to seconds.

### Example 3

The embodiment of the invention shown in Figure 4 may be used for the following protocol. The active regions of the first and second flow volumes 40b and 50a, and the first and second RF coils 28 and 26 are each connected to RF transmitter(s) and receiver(s) and tuned to the same nuclear resonant frequency. No recirculation loop is needed for this experiment. A sample is introduced into the flow vessel and flows to the region of the first coil 28. A soft pulse sequence or other specially calculated selective  $^{13}\text{C}$  pulse or RF signal is applied to the first coil 28 to excite (or, in a related protocol, to invert) individual  $^{13}\text{C}$

resonances, e.g., the resonance of a particular carbon atom in a first reactant molecule, or a selected set of resonances. Such selective pulses are well known in the art and have been described for static samples (see e.g. G.A. Morris and R. Freeman, *J. Magn. Reson.* 29, 433 (1978)). The sample then flows to a reactor located between the coils, where a second  
5 reactant is introduced. This initiates a set of chemical changes at a precise time and exact location along the flow path, which may occur instantaneously, but most preferably for the full practice of this method the reaction evolves over time according to a defined reaction mechanism or set of paths. Since the fluid is flowing the species present at a given point for an evolving reaction varies with position along the flow path. Thus, after a delay, the  
10 products (or chemical intermediates) of the reaction are detected downstream. This is done with a standard  $^{13}\text{C}$  read pulse at the second coil 26. The spectrum thus obtained will have a perturbed line, that reveals which carbon atom of the product (or chemical intermediate) molecule corresponds to the previously perturbed atom in the defined position which was spin-temperature labeled in the reactant molecule upstream, thus shedding light on the  
15 reaction pathways. The process may be repeated to trace the outcome of reaction for all atoms in the first reactant molecule. This experiment largely obviates the need for difficult and expensive specific isotopic labeling of the first reactant molecule in the study of chemical reactions. Figure 6 is a schematic view of the probe of Figure 4 showing the reaction chamber 60 located in the position 45 of the flow vessel intermediate the two coils. For the  
20 practice of this method, Figure 6 also shows a flow delay element 70 and a reactant injector 65 connected to the reactor 60 in the flow path.

The embodiment shown in Figure 6 is used for the study of a variety of chemical reactions, such as molecular rearrangements in a similar manner. The first reactant molecule passes through the first flow volume where it is excited by the first coil as described above.  
25 It then enters the reactor. Illustratively, the reactor need not introduce another chemical reactant, but may simply initiate a rearrangement or fragmentation reaction, e.g. by cooling, irradiation with ultraviolet light, or contact with an immobilized catalyst or enzyme in the reactor. After flowing through a certain length as before, the intermediates or products of the reaction are detected by the second RF coil, thus revealing information about the course of  
30 the reaction or rearrangement. The delay element provides a definite and preferably variable delay path to precisely control the time instant following initiation at which downstream detection occurs. Delay element 70 may for example consist of a telescoping U-tube in the flow path, which provides a variable path-length of fixed cross-section connecting the flow paths. The use of such a delay element results in a very dependable and repeatable  
35 mechanism for time sampling of the reactant products, since the sampling delay can be controlled without changing the transit times through either the first or the second coil.

The embodiment shown in Figure 6 is also used in yet other novel protocols. In one such technique, the first coil and the second coil are each used as both transmitters and



receivers. The first coil obtains an NMR spectrum of the first reactant according to standard protocols. The reactant then flows to the reactor where a reaction is initiated (e.g. by heat, light or the like, as described above). After flowing through the delay volume, the spectrum of newly evolving intermediates or products is detected by the second coil. A difference spectrum is then generated by subtracting the first spectrum from the second spectrum. This has the effect of canceling solvent peaks and other peaks common to both spectra, e.g., to filter spin-temperature labeled peaks. This procedure has the advantage of reducing receiver dynamic range problems associated with large solvent peaks. Provided the volumes within the coils and the fields are sufficiently well matched, the differencing operation may be performed on intermediate or transformed data sets rather than fully analyzed spectra, greatly reducing computational load and/or storage requirements. For such operation, rather than the dissimilar tubing shown for the upper flow chamber shown for Figure 6, a flow chamber identical to the lower one would be provided. To facilitate such configurations, flow chambers, reaction vessels and delay portions and the like may be provided in modular, snap-together embodiments.

The invention further contemplates a probe as shown in Figure 11 including a flow chamber having two NMR detection regions, and further including a premagnetization volume and a reaction chamber, both upstream of both coils, and a delay path intermediate the two coils. In Figure 11 a premagnetization volume 80 is provided in the flow region 40, and a reaction chamber 60 is included in the flow volume 40 upstream of the first coil. The probe also includes a delay element 70 and a second detection coil. An exemplary protocol for operation of this probe follows in Example 4, after a general discussion of the additional elements of the premagnetization volume and reaction chamber.

For many measurements, the attainment of maximum sensitivity requires the sample nuclei to be pre-equilibrated in a region of high magnetic field to establish the Boltzman distribution of spin states. It is therefore important to provide the sample with sufficient residence time in the high magnetic field before NMR measurements begin. The premagnetization volume is preferably made of a nonmagnetic material, for example Teflon or Pyrex. One premagnetization volume suitable for allowing such residence time is the upper chamber already shown in the region of the upper coil in Figure 6, which may perform this function when operation of the upper coil is not required. In that embodiment, a length of Teflon tubing 75 is wound around a spool. This construction allows one to select a residence time in the field by selecting an appropriate size and length of tubing, while arranging that the fluid arrives as a bolus with substantially uniform residence characteristics. Another exemplary embodiment employs a Pyrex flow vessel having a large diameter relative to the diameter of the flow vessel immediately upstream to augment residence time. In still another embodiment, the flow vessel comprises an outer tubular member and an inner tubular member, fitted together with a fluid-tight seal, sharing an axis and having a length along that

axis. One tubular member is movable relative to the other, and the volume within the vessel may be varied by sliding the movable member along the shared axis, much like the telescoping delay path configurations described above. This construction capitalizes on the increased volume of the delay section, without any changes in coil or magnet shims, but does not necessarily utilize the available space in the high field very efficiently.

In the context of the present invention, the illustrated reaction chamber is a small flow volume of the flow vessel, preferably constructed of a nonmagnetic material, arranged to produce turbulent mixing or intimate and complete contact or treatment of the sample as it passes through the chamber. In certain embodiments, the reaction chamber will have fittings by which flow tubing for the sample and a reagent or reagents may be connected. The reaction chamber may also be constructed with a temperature-control arrangement such as a heater. In certain embodiments, the reaction chamber may be equipped to initiate or facilitate or complete a process by irradiation, by using, for example, ultraviolet light or a laser light source. One reaction chamber suitable for use in the probe of the present invention has been described in U.S. Patent 5,198,766, of inventors M. Spraul and M. Hofmann.

While not shown in Figure 11, a reentrant flow path such as shown in phantom in Figure 4, may be useful to provide recirculation of the fluid flow from a downstream region to an upstream region, thus forming a closed loop, allowing multiple passes through a region, before or during a measurement. Fluid may be made to flow in the loop 62 by a pump which may be pneumatically powered, preferably by an air source such as is commonly present in NMR machines for spinning sample turbines in the magnet assemblies. The pump is made of nonmagnetic materials, and is preferably a peristaltic or impellor pump.

#### Example 4

In a representative protocol for the probe of Figure 11, the fields and chambers in the active regions of the first and second flow volumes A and B are well matched, and the coils 26 and 28 are also well matched. A sample is introduced at a position upstream of the flow region and flows into the premagnetization volume 80 in the static magnetic field. The volume of chamber 80 is sufficiently large that the residence time of a flowing bolus of fluid is long compared to  $T_1$ , insuring adequate premagnetization and thus maximum sensitivity of detection. The sample then flows to the reaction chamber 60, where a process is initiated or a reactant is introduced. This initiates a set of chemical changes which evolve over a period of time. The sample flows on, to the first detection region A, where a first NMR spectrum is obtained by coil 28 by a conventional method. The sample then flows to the delay element 70, where the reaction is allowed to further evolve for a defined period. The sample then flows to the second detection region B, where a second NMR spectrum is obtained, using

signals on coil 26. Thus, the same measurement conditions are applied to a single sample which has evolved as it flows between coils. In a basic embodiment of this aspect of the invention, the spectrum obtained at the first coil 28 is subtracted from the spectrum obtained at the second coil 26. The resulting difference spectrum reveals only those components of the spectrum (lines or line amplitudes) which have changed during the reaction course. The difference procedure also effectively cancels the solvent and other large constant peaks, such as those due to excess reagents, which can be a significant factor in reducing dynamic range difficulties when the desired resonances are weak. Further, the time delay introduced by the delay element 70 may be changed in a systematic manner, allowing the study of the reaction course over time. This time delay may be used as one dimension of a two-dimensional experiment, as described elsewhere. Further, provided the two coils, chambers and surrounding field conditions have been matched sufficiently closely, the difference procedure may be applied to intermediate blocks of data or data transforms, thus greatly reducing the volume of data processing or storage required to obtain useful spectra.

Returning to the description of the time delay path element 70, it will be understood that a variety of structures can be used to vary the length of time between when the volume of the fluid is irradiated in the upstream coil and when it is finally presented within the downstream coil. Figure 12, for example, illustrates an embodiment of a delay element 70' which provides a set of telescoping U-tubes 100 which can be disposed in the flow path as part of the flow vessel 22. The length of the path between inlet and outlet 102A, 102B of the nested U-tubes 100, with consideration of the flow velocity, defines the delay time provided by delay element 70'. Accordingly, as illustrated in Figure 12, extension (or compression) of the telescopic portion 104 between each of the U-tubes 100 varies the length of the flow path through the delay element 70' and, when the flow velocity is constant, permits incremental increases (or decreases) in the time it takes for a bolus of sample conditioned in the upstream coil to reach a downstream position for further mixing and detection.

Figure 13A and 13B illustrate an embodiment of a delay element 70" which provide a set of nested, telescoping U-tubes 100' which permit radial expansion of the delay element in the intercoil region, thereby making use of space within the probe without reconfiguring the coils or magnets. In one embodiment of the delay element of Figure 13A, the tube support members 106 are moved relative to the axis of the telescopic portion 104 by an air or fluid actuated mechanism.

In yet another exemplary embodiment, delay element 70 can be provided in a plurality of valves for switching tubing of different lengths in and out of the intercoil flow path of fluid vessel 22. For instance, in one embodiment a carousel can be supplied which comprises individual coils of different lengths of tubing. The carousel is rotated to couple an inlet and outlet of a given tubing as part of the fluid vessel 22 within the intercoil space by a fluid-tight

coupling. In other embodiments, tubing of various length can be connected and provided in the flow path of fluid vessel 22 by the use of selectively actuated pinch-valves.

There are a number of different reasons Applicants provide a time-delay, e.g., by variable volume or flow path length, in the flow path of the intercoil space for both one-dimensional and multi-dimensional NMR experiments. As indicated in one example above, by balancing the flow rate with the duty cycle on the pulse generator (e.g. periodicity of nutation pulses) and other acquisition parameters one can optimize sensitivity of a flow measurement. Moreover, in the instance of those embodiments which provide a reaction chamber, the inclusion of time-delay element which can be varied by the operator, as shown in Figure 6, can permit the acquisition of spectra at incremental time points after a reaction has been initiated. For instance, the dimensions of the time-delay element can provide a time-delay in the range of milliseconds to resolve the formation or fate of short-lived intermediates..

In another embodiment, the time delay feature of the subject probe can be used to generate two-dimensional (or multi-dimensional) NMR spectra. An important strength of modern NMR spectroscopy is the ability to drive the dynamics of the spin system through a series of coherence transformations such that the resultant observable magnetization reflects desired combinations of evolutions and interactions. Coherence transformations can be used not only for transferring magnetization from one spin to another (such as described in detail above), but also for "filtering" purposes to select only those spin systems with a given property. Coherence transformations can be used, for example, in a two-dimensional (2D) NMR experiment to display correlations between coupled spins. This can be accomplished in the subject probe by exciting coherences with a preparation pulse sequence at the upstream coil, then allowing them to evolve for a variable time  $t_1$  (Figure 14) which is the time it takes the conditioned fluid to flow from the upstream to the downstream coil. After the evolution period, a mixing pulse sequence is applied to the system by application of one or more nutation pulses at the downstream coil, followed by observation at the downstream coil of the (-1)-quantum coherences during the period  $t_2$ . The resulting FID signal is processed in a manner similar to the one-dimensional experiment by taking the Fourier transformation of the signal. However, a second time dimension is introduced by repeating the experiment while systematically varying the length of the evolution time period  $t_1$ , e.g. by varying the time it takes for a fluid sample to move from the upstream coil to the downstream coil. The resulting set of FIDs are then subject to a second Fourier transformation over the varying time of the evolution period. The two frequency spectra can be plotted along the horizontal and vertical coordinates of a graph to produce a two-dimensional plot. This plot has "peaks" which represent coherence transformations and, therefore, is useful in studying, for example, molecular structures as it can contain information pertinent to the three-dimensional geometry of the molecule.

Coherence transformations can be used, for example, in homonuclear two-dimensional (2D) NMR experiment to display correlations between coupled protein spin systems. A typical proton homonuclear 2D experiment involves the interaction of each proton ( $^1\text{H}$  nucleus) with all of its coupling partners, such as through-space or through-bond couplings. In an exemplary measurement protocol, the protons are excited during the preparation time period by an application of an RF nutation pulse at the proton resonant frequency through the upstream coil. The resulting coherence evolves under influence of the chemical shift during the  $t_1$  evolution time period as the conditioned fluid flows through the fluid vessel in the intercoil space. The coherence is then transferred between coupled protons during the mixing period by applying a second RF pulse to the protons via the downstream coil. Finally, during a detection period, the FID is sampled at the downstream coil. The resulting FID signal is modulated not only by the chemical shift evolution of the protons during the detection period but also by the spin evolution period of coupled protons. Such experiments include CORrelation SpectroscopY (COSY) and NOE Spectroscopy (NOESY) experiments. For instance, in COSY experiments and other through-bond sensitive experiments, correlation of (-1)-coherence through J-coupling can be used to establish that the coherences belong to the same coupling network. Other homonuclear experiments, as well as the appropriate configuration of the subject probe, will be apparent to those skilled in the art in light of the present disclosure.

Similar multi-dimensional experiments can be performed in heteronuclear embodiments of the subject probe, as for example, 2-dimensional NMR experiments specifically designed for the detection of proton-coupled  $^{15}\text{N}$  or  $^{13}\text{C}$  chemical shifts. For instance, polarization transfer experiments based on an INEPT or reverse-INEPT pulse sequence between the upstream and downstream coils, such as a reverse Heteronuclear Multiple Quantum Coherence (HMQC) 2-D experiment, can be used to make resonance assignments.

The invention being thus disclosed and the operation of representative embodiments described, variations and modification of the probe apparatus, and a wide range of homo- and heteronuclear measurement techniques which can be performed with one or more different embodiments of the subject probe will be apparent to those skilled in the art in light of the present disclosure, and all such variations, modifications and embodiments are considered to be within the scope of the present invention, as set forth in the claims appended hereto.

What is claimed is:

1. A probe for holding a sample of material in a test region of high magnetic field and uniformity in an NMR apparatus, such probe comprising:
  - 5 a housing for positioning a sample-holding body in a test region and a body secured to said housing, said body having a flow chamber with an opening for introducing fluid to flow along a flow path through said test region, the flow chamber comprising in flow sequence
    - a first flow volume disposed in a first region
    - 10 a second flow volume disposed in a second region downstream of and adjacent to the first flow volume
    - so that flow through said first and second regions experiences the high field of said test region:
    - a first coil disposed in said first region and tunable to a first nuclear resonant frequency;
    - 15 a second coil disposed in said second region and tunable to a second nuclear resonant frequency;
    - whereby excitation of said first coil when a sample flows through said flow chamber enhances nuclear resonance detected by said second coil.
- 20 2. A probe according to claim 1, further comprising flow recirculation means in said body for providing a return flow path from said second to said first flow volume.
3. A probe according to claim 1, further comprising a microwave cavity in said body
- 25 located in said first region.
4. A probe according to claim 3, wherein the first flow volume of the flow chamber includes means for immobilizing a free radical to receive microwave energy in said cavity and couple spin to nuclei flowing therethrough.
- 30 5. A probe according to claim 3 or 4, wherein the first coil spin locks nuclei as they traverse the microwave cavity.
6. A probe according to claim 1, wherein the first and second coils each include a proton
- 35 decoupling winding operable to suppress splitting during NMR detection through the coils.
7. A probe according to claim 1, wherein the first coil is tunable to spin-mark heteronuclear atoms, and wherein said flow chamber further comprises a reactor between said first and said second regions for initiating a reaction such that an analyte with a marked

heteronuclear atom undergoes a molecular change whereby heteronuclear detection at said second coil detects new molecular bonds of the marked atoms.

- 5 8. A probe according to claim 2, wherein said flow recirculation means further comprises a fluid-driven pump.
9. A probe according to claim 1, wherein said first and second flow volumes of said flow chamber have different cross-sectional areas.
- 10 10. A probe according to claim 9, wherein said cross-sectional areas differ by a factor greater than ten.
11. A probe according to claim 1, wherein said first and second flow volumes of said flow chamber define different flow axes.
- 15 12. A probe according to claim 3, wherein said first coil and said microwave cavity are effective to apply mutually orthogonal fields to material flowing in said first region.
13. A probe according to claim 1, wherein said flow chamber further comprises delay means for varying flow time between said first and said second regions, without changing a flow rate entering said first region.
- 20 14. A probe according to claim 13, wherein said delay means includes a telescoping conduit intermediate said first and said second flow volumes.
- 25 15. A probe according to claim 13, wherein said delay means includes chromatograph column packing material held in said chamber upstream of said second flow volume.
16. The probe of claim 1, wherein said first coil and said second coil are tuned to the same nuclear resonant frequency.
- 30 17. The probe of claim 1, wherein said first and said second coil are tuned to different nuclear resonant frequencies.
- 35 18. The probe of claim 1, wherein at least one of said first coil and said second coil are selected from the group consisting of a Helmholtz coil, a Golay coil, a saddle coil, and a toroidal coil.

19. A probe for holding a sample of material in a test region of high magnetic field and uniformity in an NMR apparatus, such probe comprising:

5 a housing for positioning a sample-holding body in a test region and a body secured to said housing, said body having a flow chamber with an opening for introducing fluid to flow along a flow path through said test region, the flow chamber comprising in flow sequence a first flow volume disposed in a first region and a second flow volume disposed in a second region and connected immediately downstream of the first flow volume, so that flow through said first and second regions experiences the high field of said test region;

10 a first coil disposed in said first region and tunable to a first nuclear resonant frequency;

a second coil disposed in said second region and tunable to a second nuclear resonant frequency;

a microwave source disposed about said first coil:

15 a source of unpaired electrons disposed within said first coil;

whereby excitation of said unpaired electrons by said microwave source in combination with excitation of said first coil when a sample flows through said flow chamber enhances nuclear resonance detected by said second coil.

20 20. A method of performing an NMR measurement on a sample, such method comprising the steps of

placing the sample in a flow path defined within to a probe having a flow chamber with first and second RF coils disposed thereabout at successive upstream and downstream positions, respectively, along the flow path:

25 placing the probe in a magnetic field;

exciting the sample at one of said upstream and downstream positions; and measuring a nuclear magnetic resonance spectrum at the other of said upstream and downstream positions.



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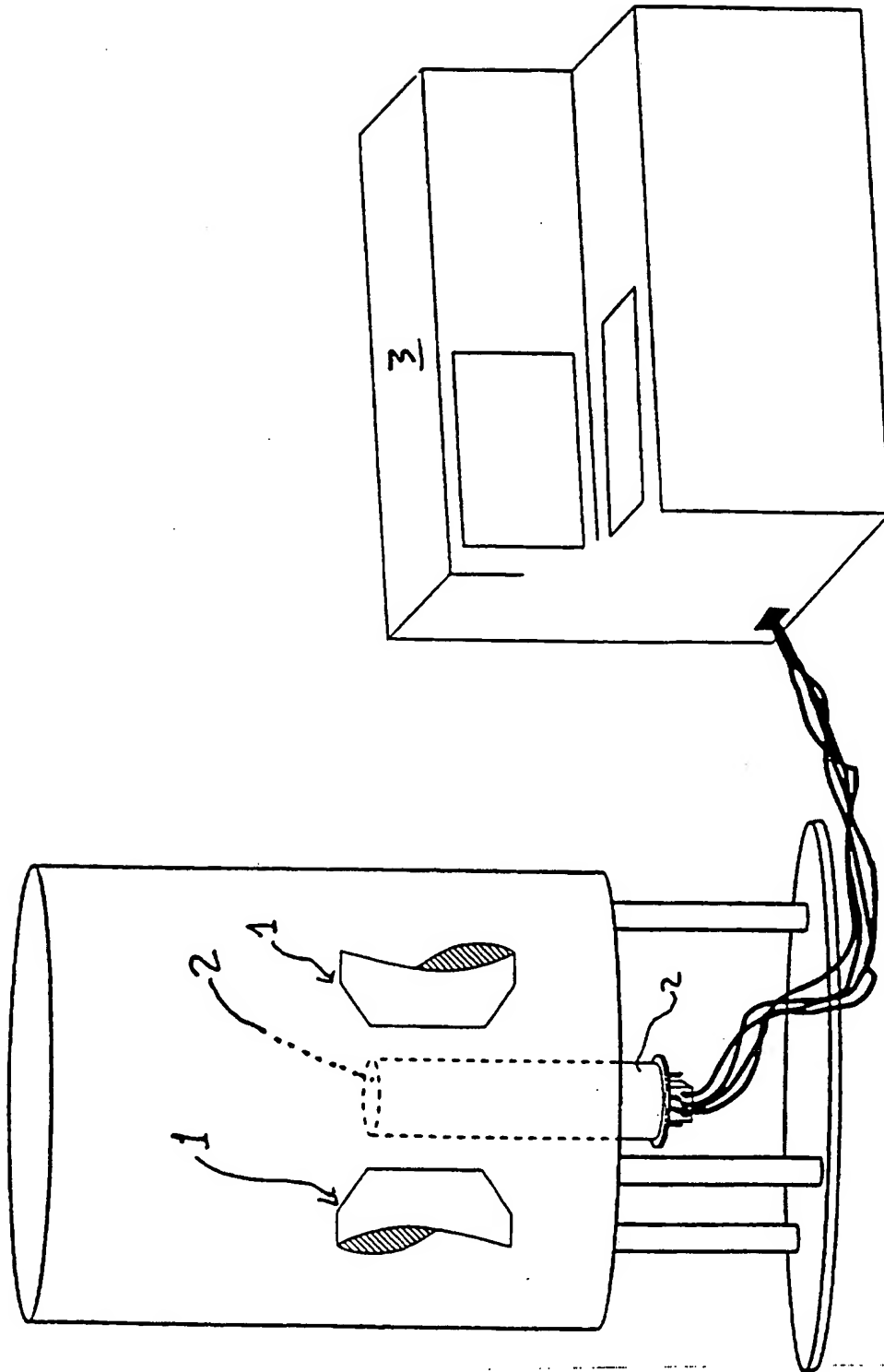


FIGURE 1

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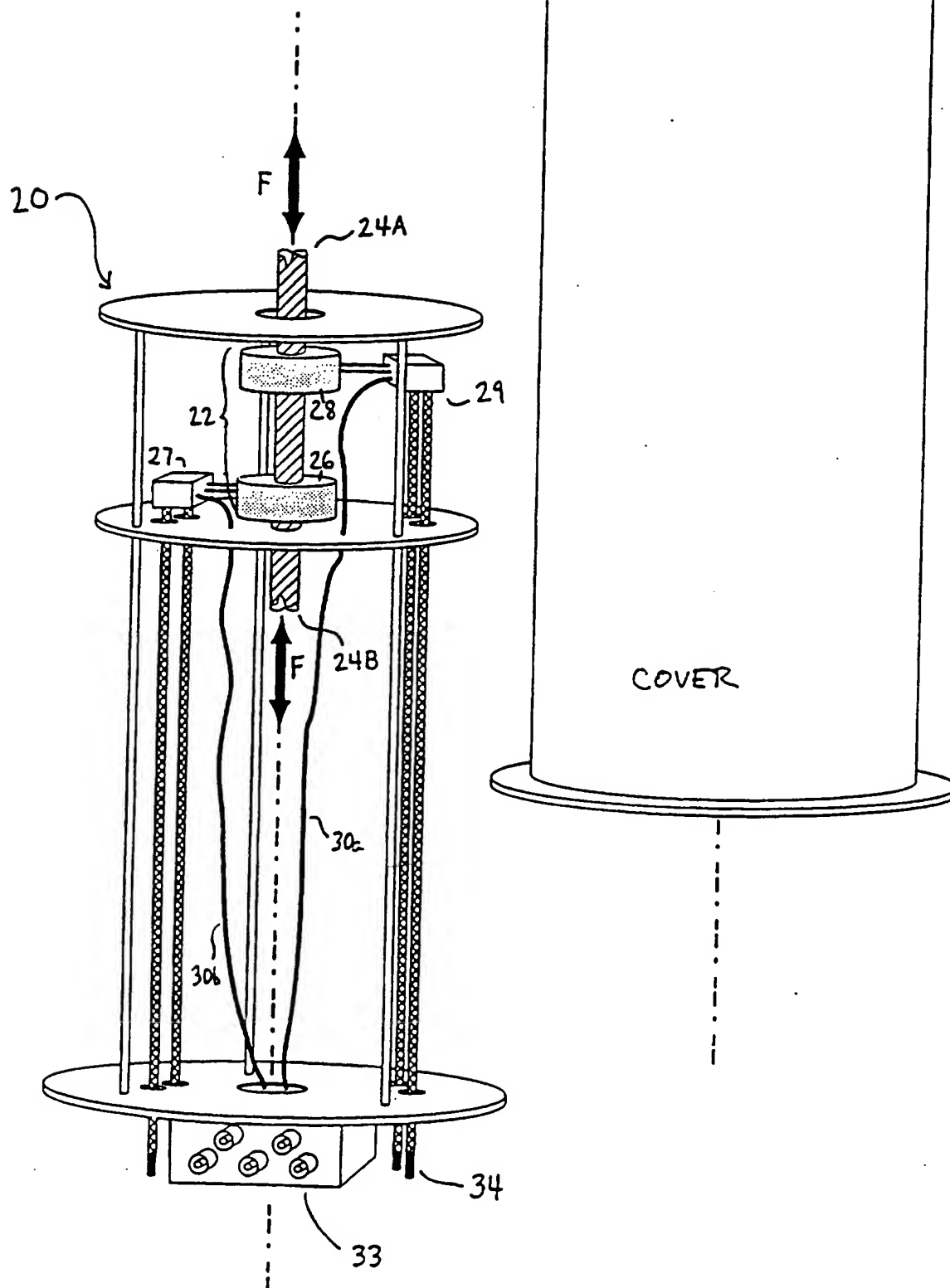


Figure 2

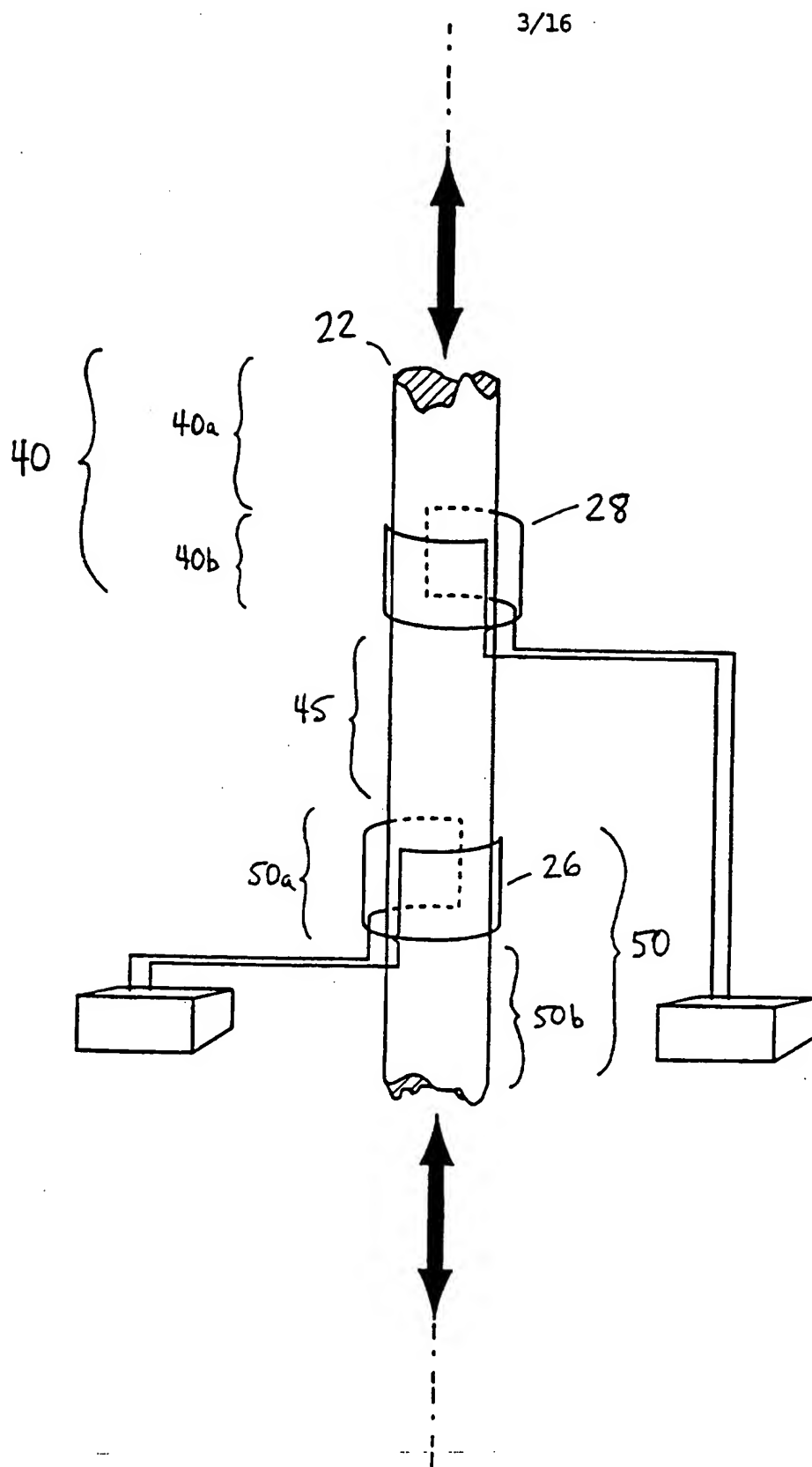


Figure 3

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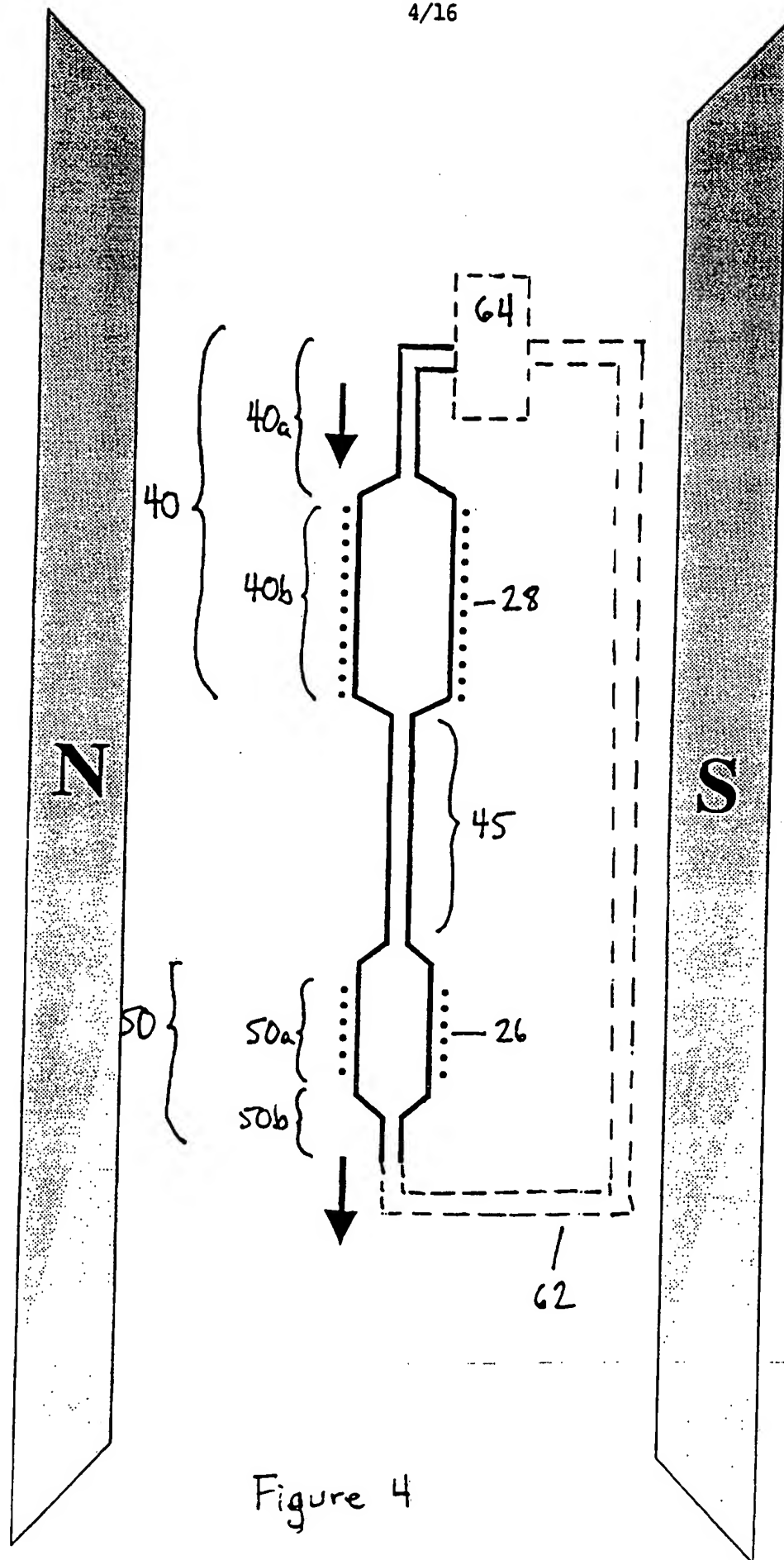


Figure 4

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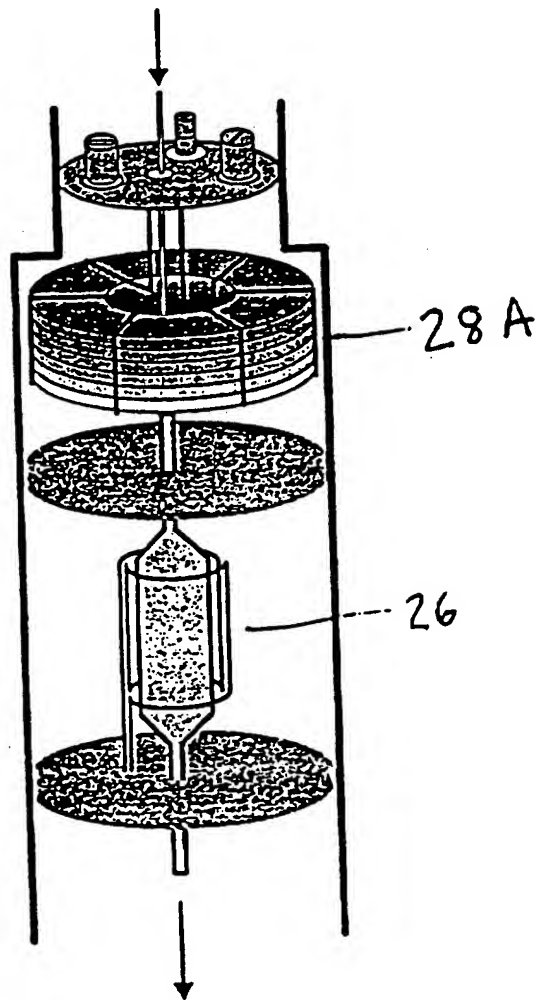


Figure 4A

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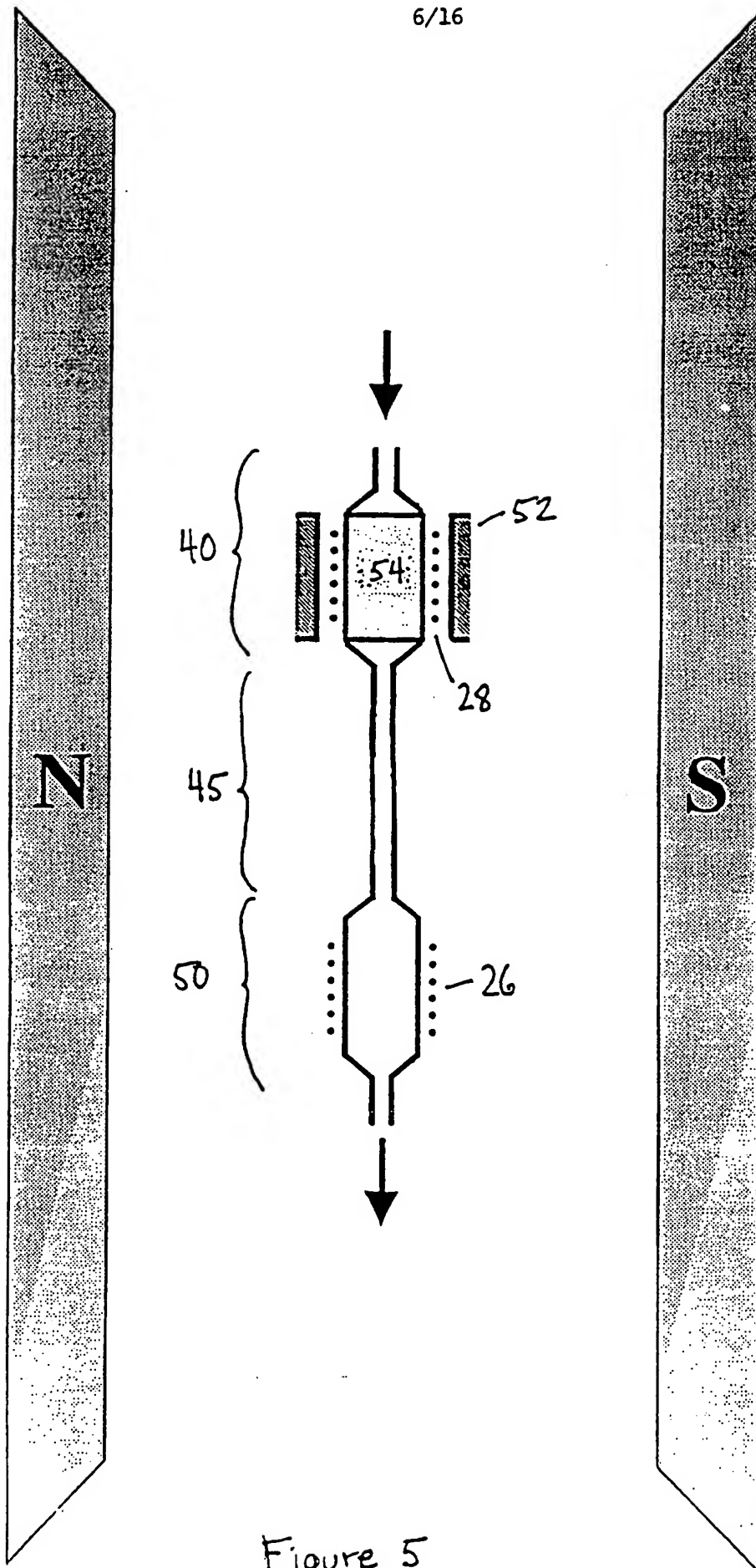


Figure 5

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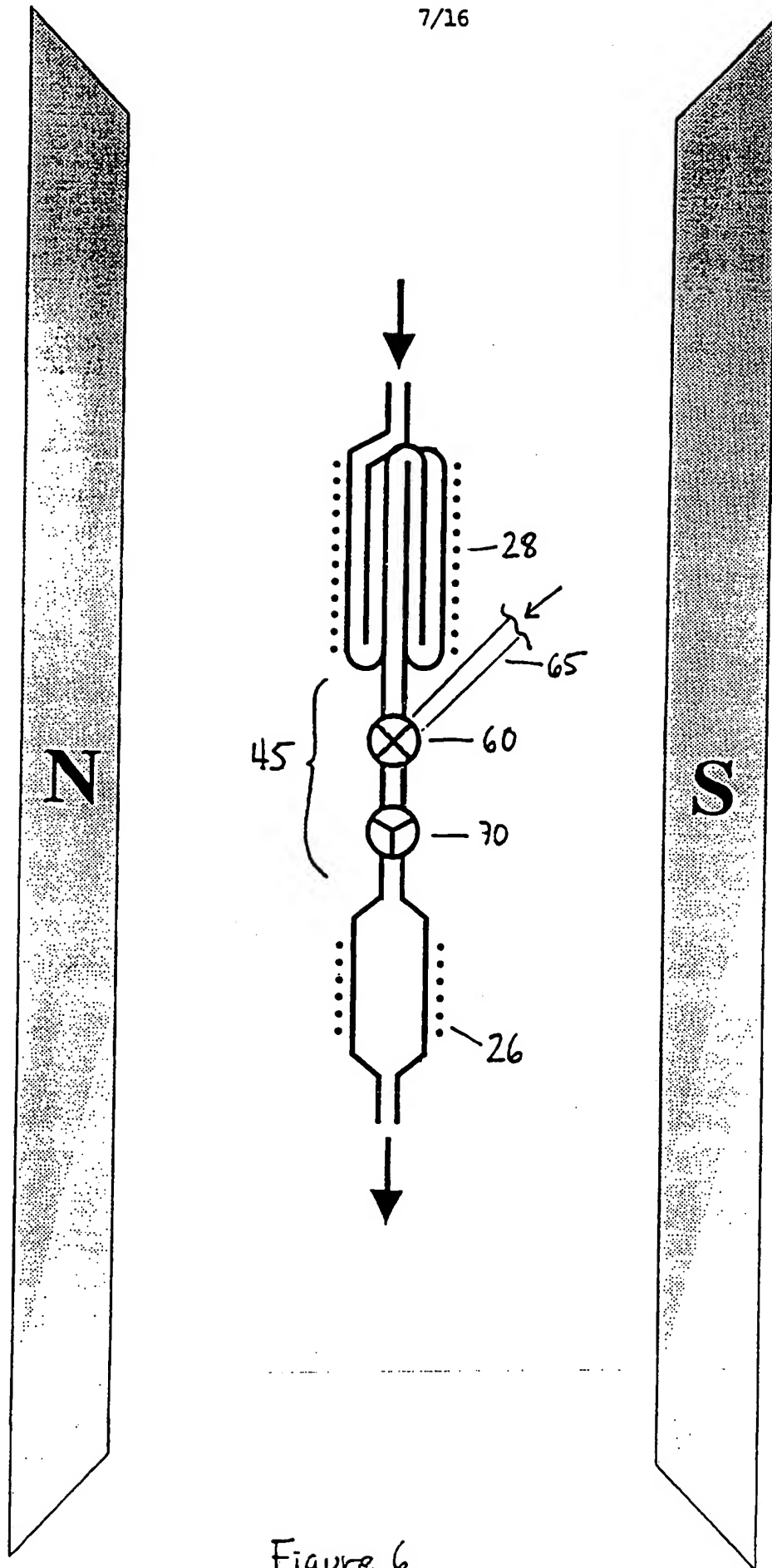


Figure 6

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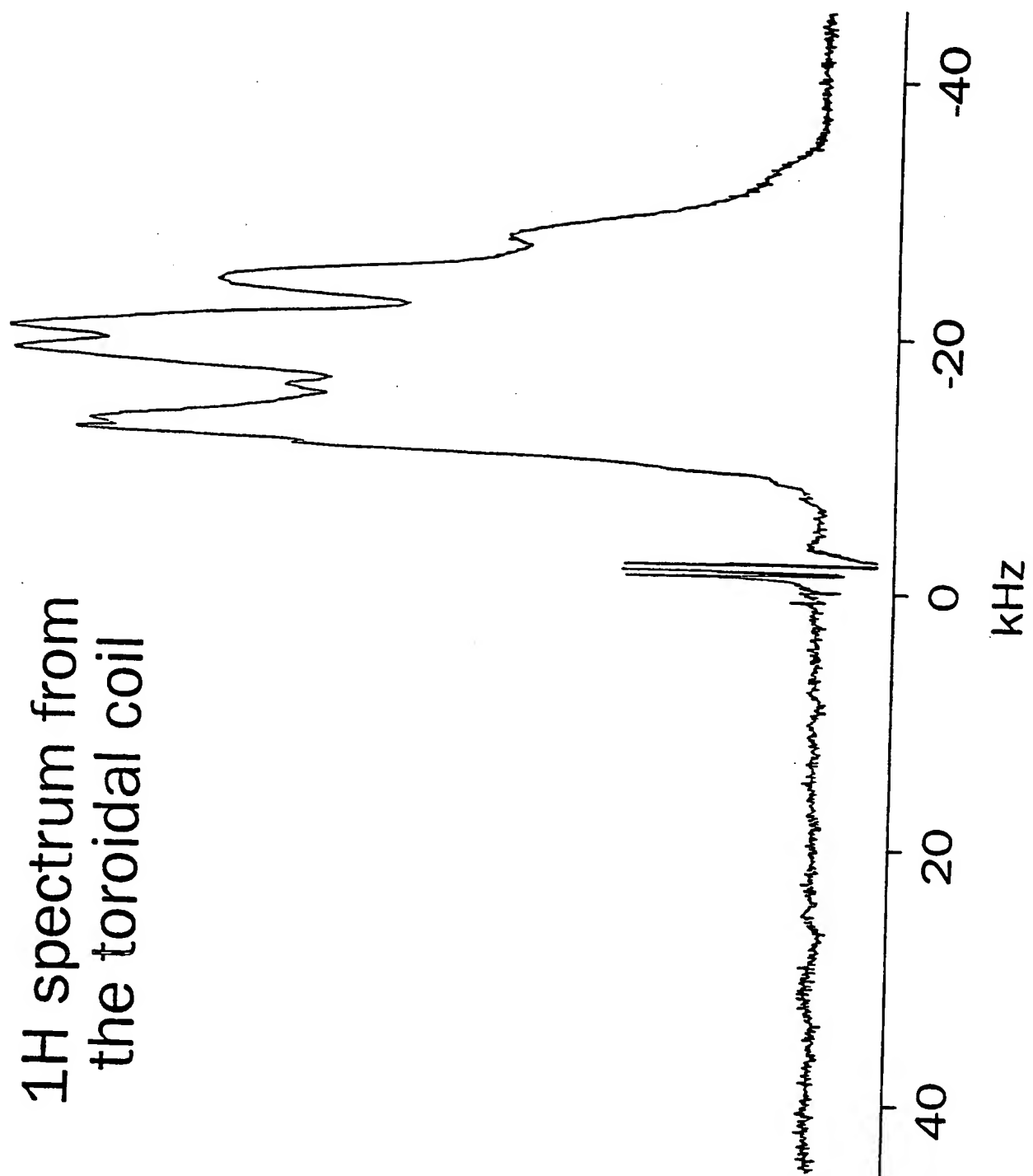


Figure 7



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## Sensitivity

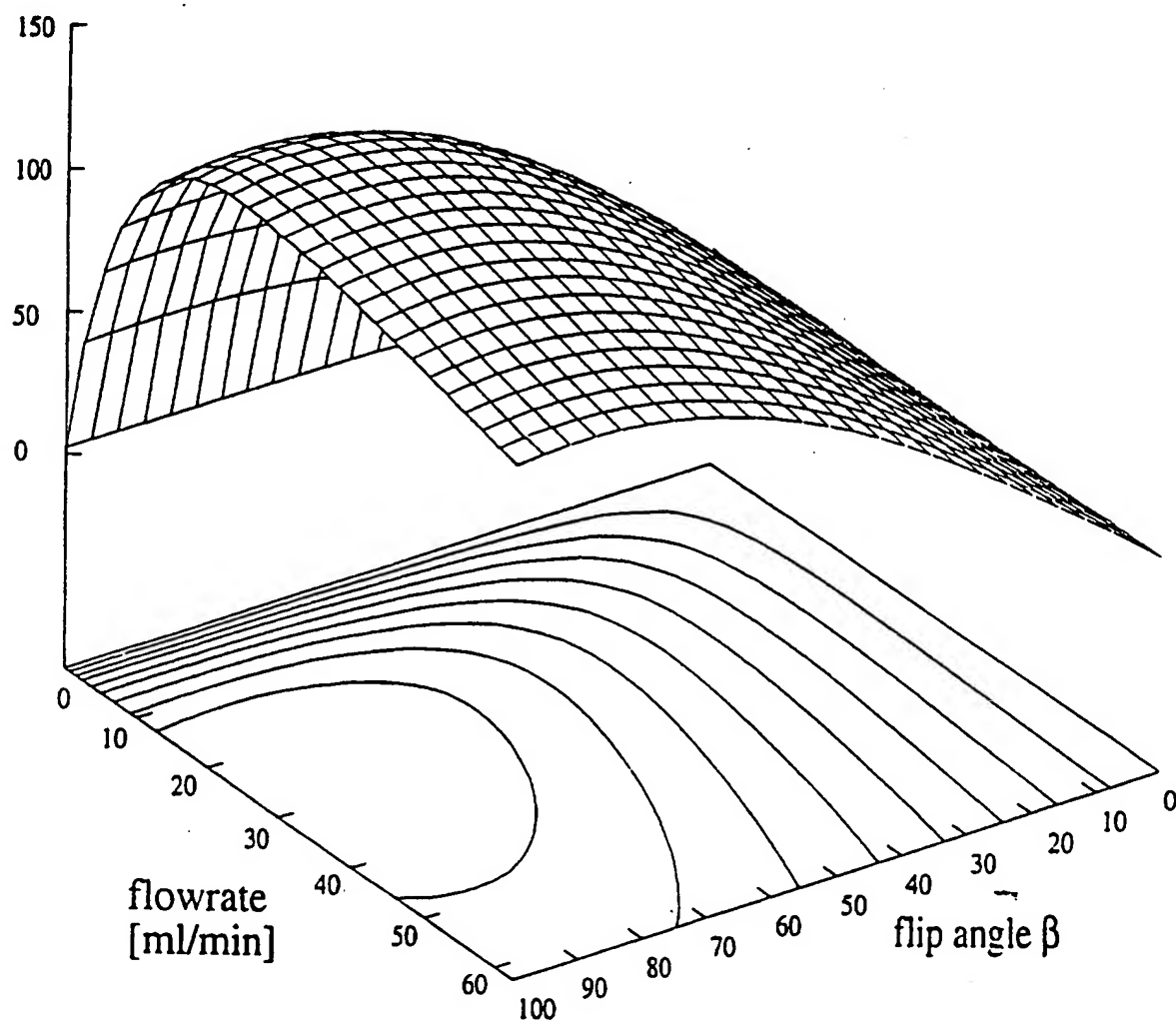


Figure 8

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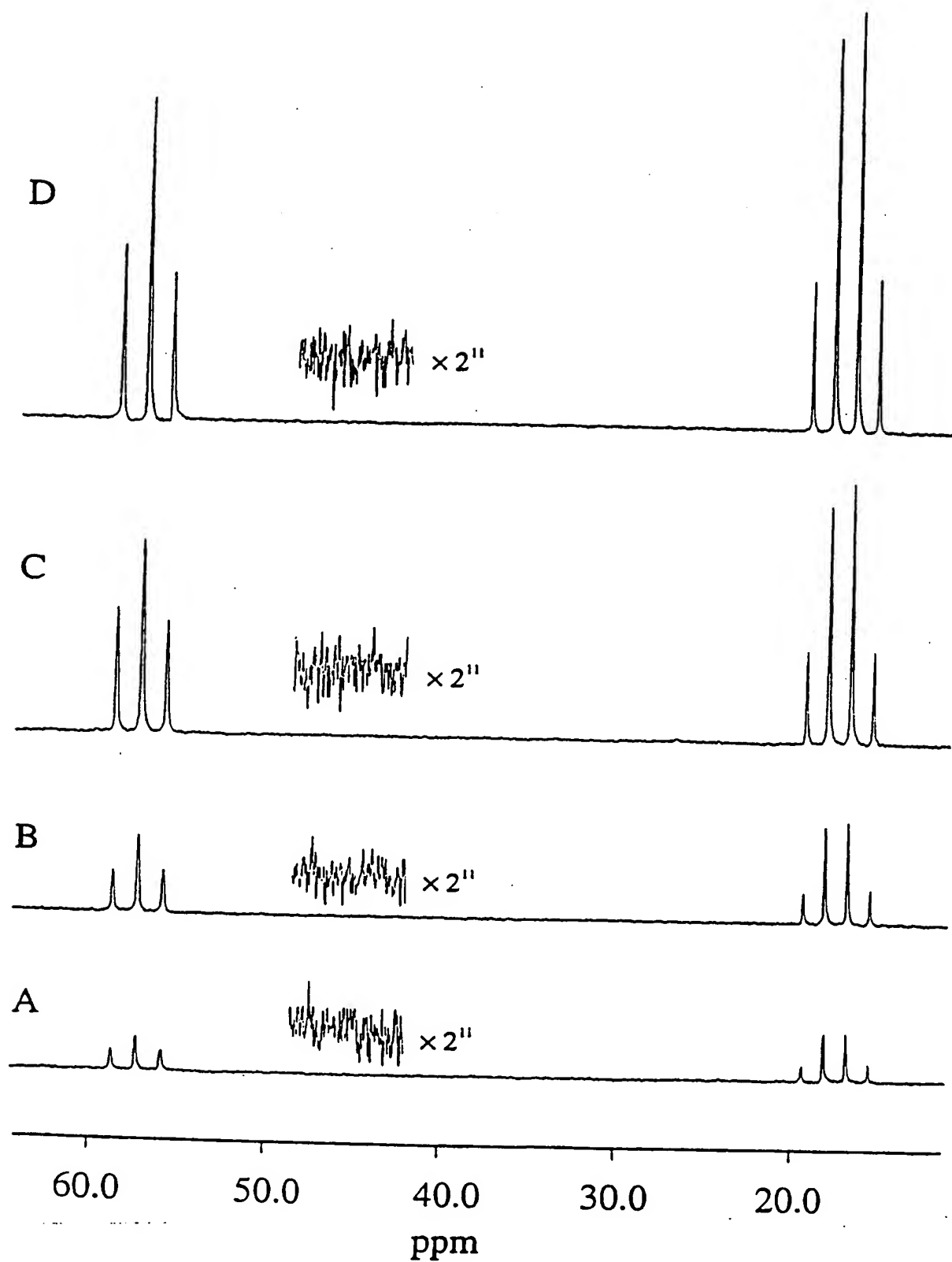


Figure 9

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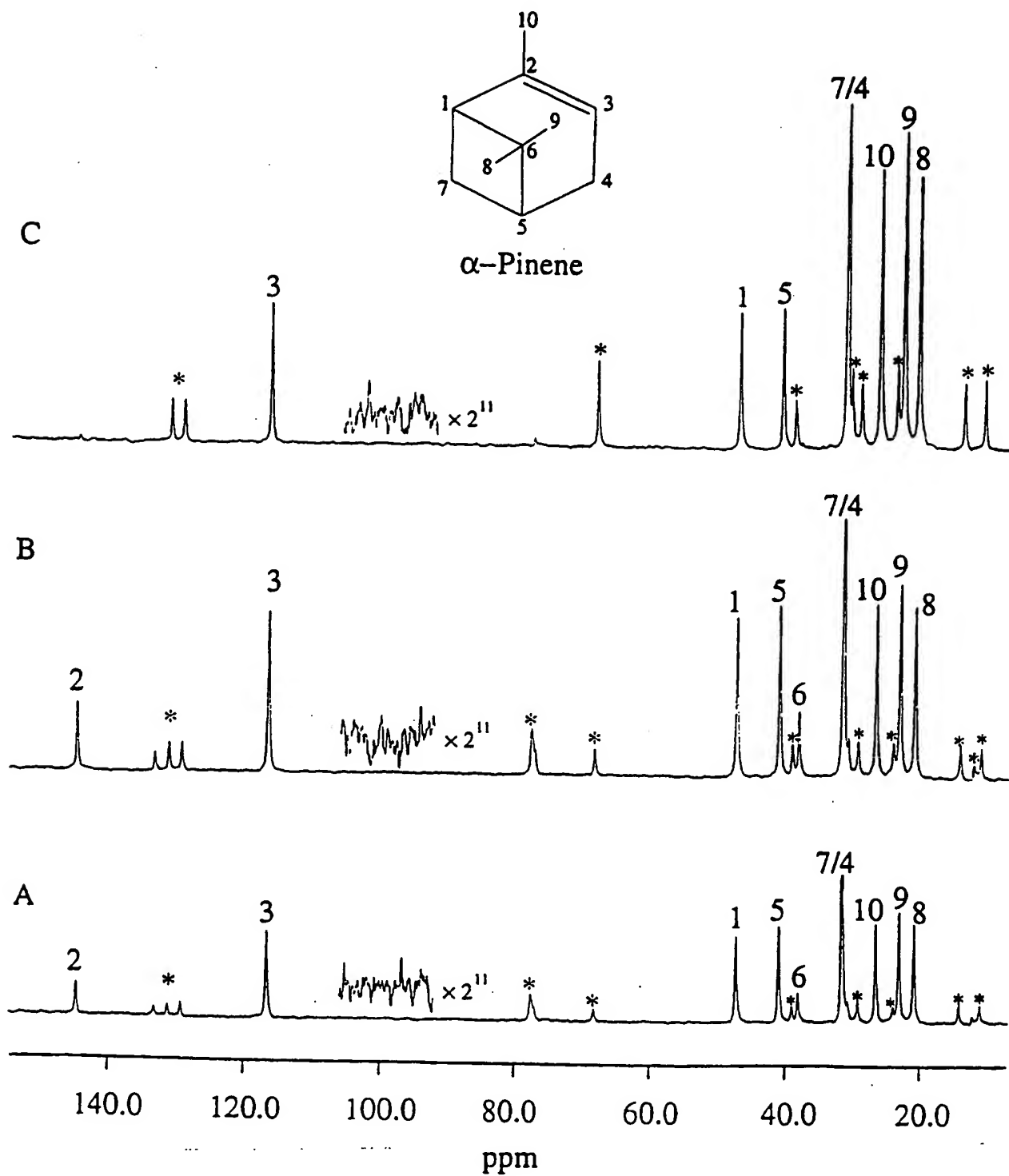


Figure 10

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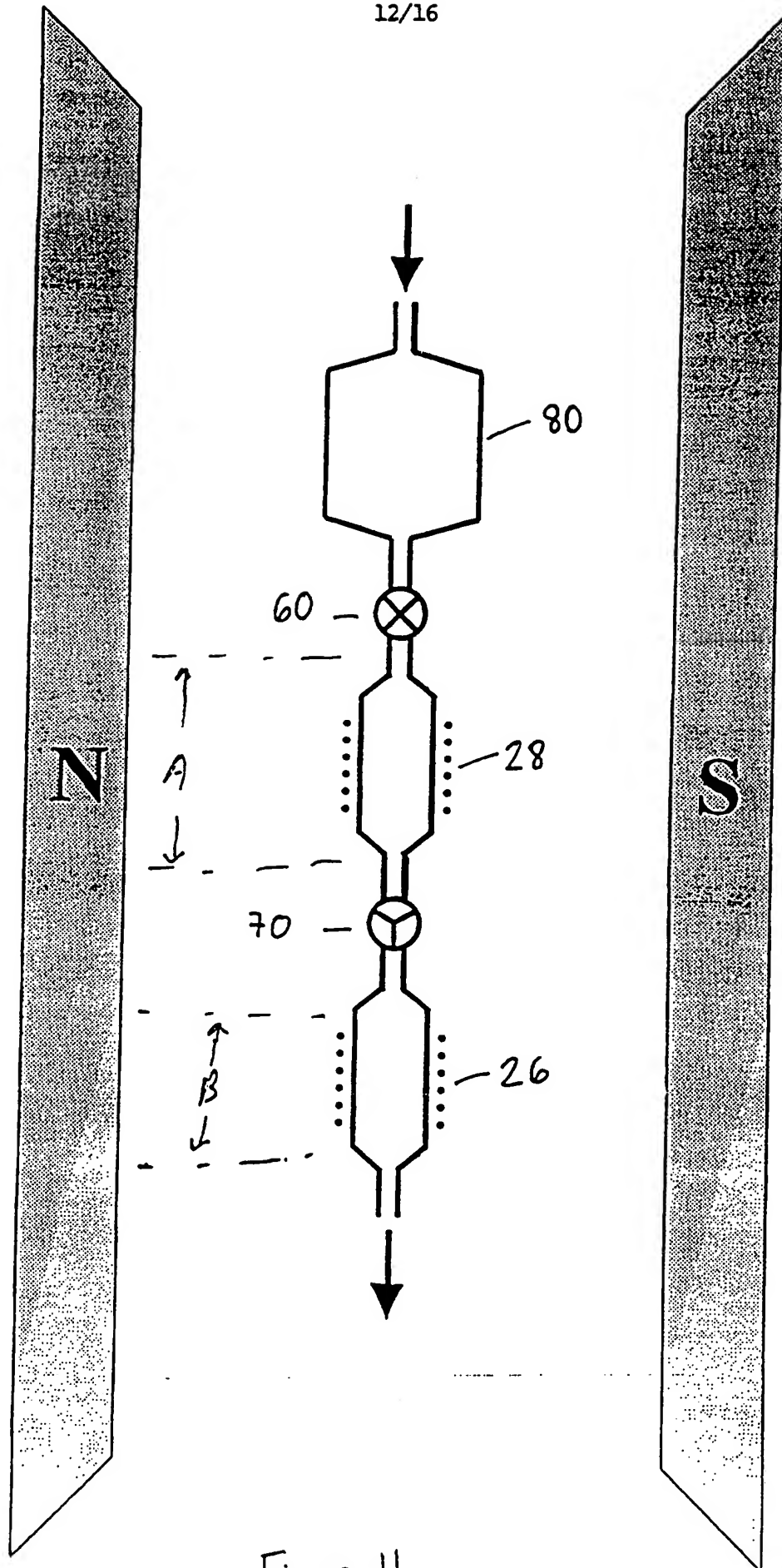


Figure 11

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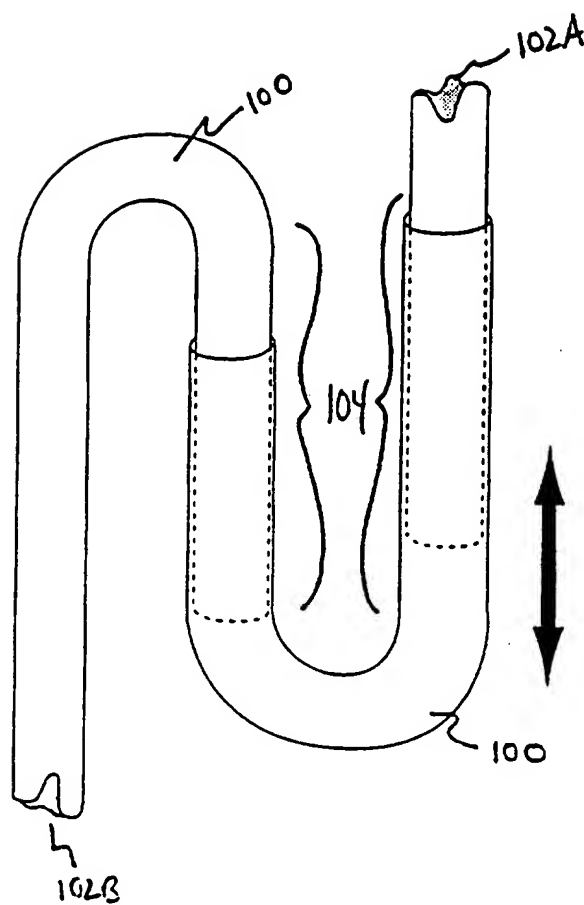


Figure 12

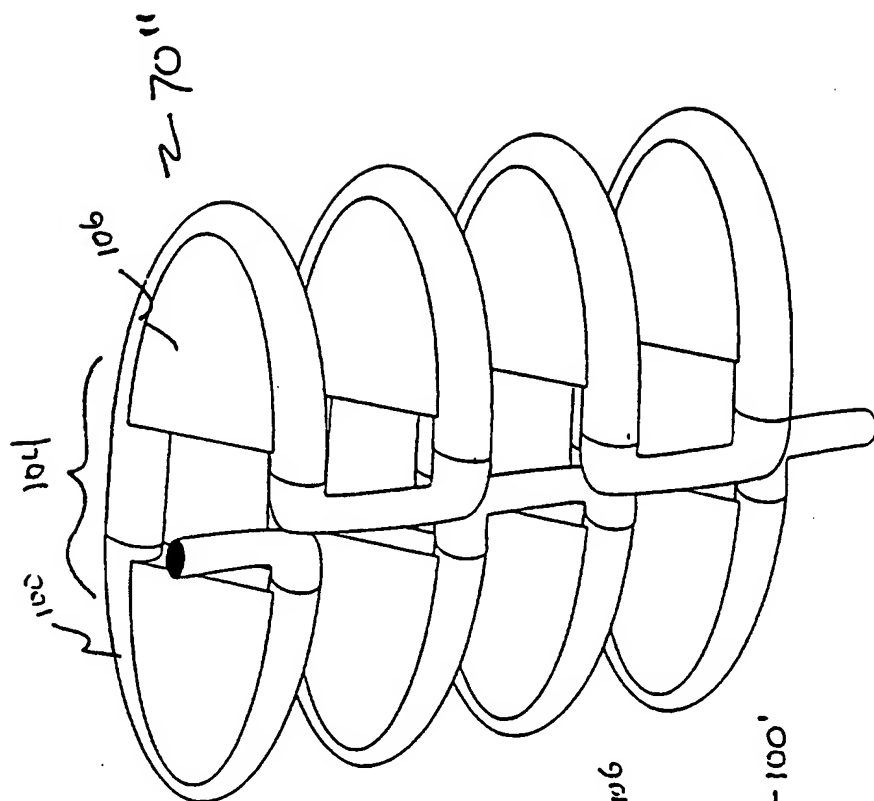
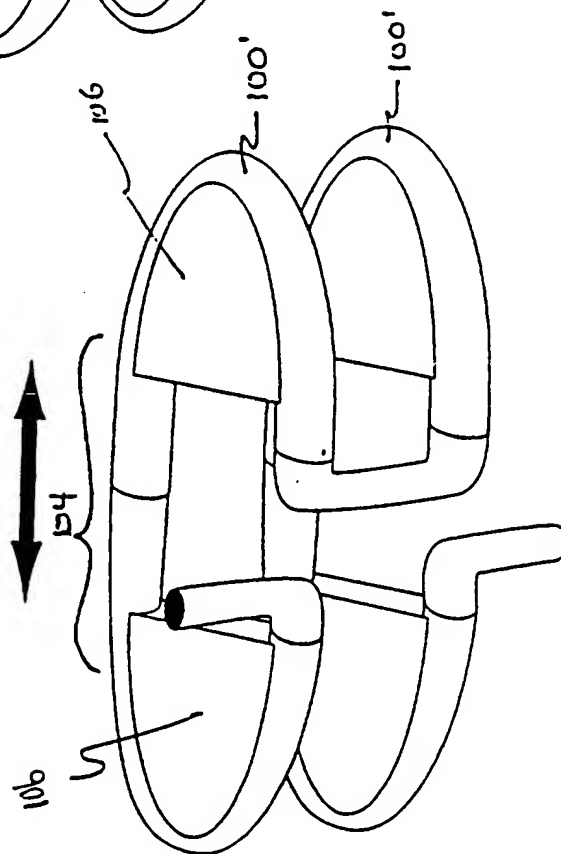


Figure 13A

Figure 13B



15/16

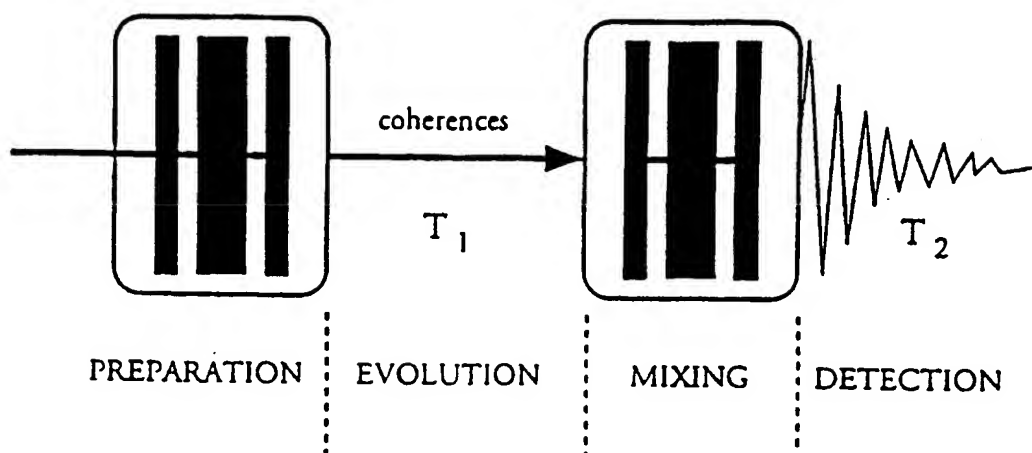
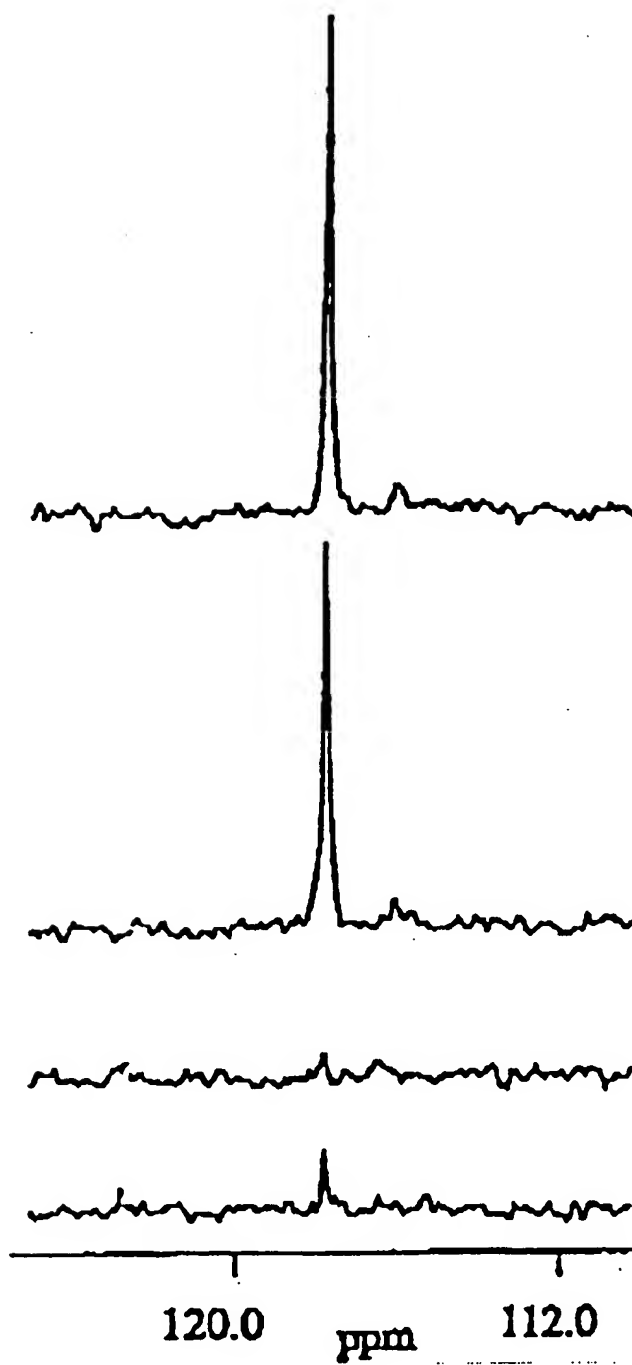


Figure 14

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*Figure 15*



**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : GO1V 3/20, 3/28

US CL : 324/300, 303, 307, 309, 310, 311, 312, 313, 314, 318, 322

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 324/300, 303, 307, 309, 310 311, 312, 313, 314, 318, 322

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,629,987 (KING ET AL) 16 December 1986	1-20
A	US, A, 4,638,251 (KING) 20 January 1987	1-20
A	US, A, 4,531,093 (ROLLWITZ ET AL) 23 July 1985	1-20
A	US, A, 5,352,979 (CONTURO) 04 October 1994	2, 6-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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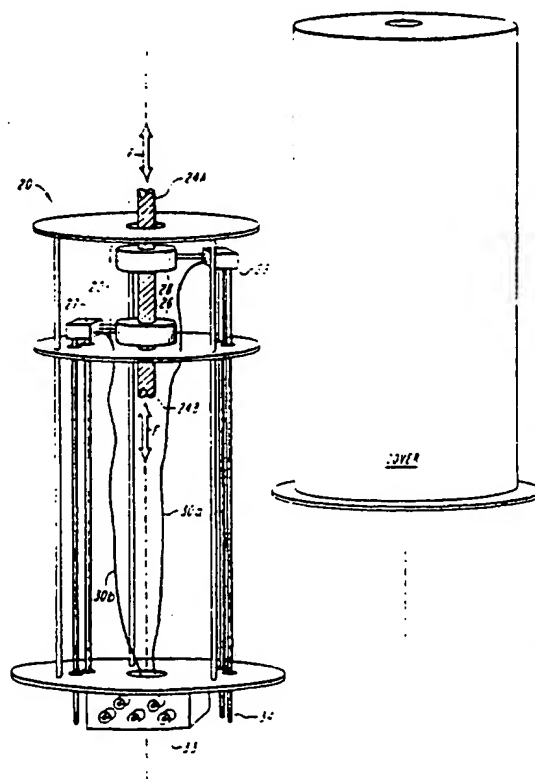
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: <b>PCT/US95/13974</b></p> <p>(22) International Filing Date: <b>26 October 1995 (26.10.95)</b></p> <p>(30) Priority Data: <b>08/329,715</b>      <b>26 October 1994 (26.10.94)</b>      <b>US</b></p> <p>(71) Applicants: <b>TRUSTEES OF TUFTS COLLEGE [US/US];</b> Tufts University, Ballou Hall, Medford, MA 02155 (US). <b>EBERHARD-KARLS-UNIVERSITAET TUBINGEN</b> [DE/DE]; Wilhelmstrasse 5, D-72074 Tübingen (DE).</p> <p>(72) Inventors: <b>SUDMEIER, James, L.;</b> 50 Village Brook Lane #8, Natick, MA 01760 (US). <b>GÜNTHER, Ulrich, L.;</b> 21 Harlow Street, Arlington, MA 02174 (US). <b>BACHOVCHIN,</b> William, W.; 71 Warwick Road, Melrose, MA 02176 (US). <b>ALBERT, Klaus;</b> Hinebuchenweg 25, D-72076 Tübingen (DE).</p> <p>(74) Agents: <b>VINCENT, Matthew, P. et al.;</b> Lahive &amp; Cockfield, 60 State Street, Boston, MA 02109 (US).</p>	<p>(81) Designated States: <b>AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</b></p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

(54) Title: **TANDEM COIL NMR PROBE**

## (57) Abstract

An NMR probe (20) positions a flow chamber (22) with first and second flow regions in the high field of an NMR apparatus. A second, downstream, flow region is surrounded by an exciter/detector coil which may be of a conventional type for home- or heteronuclear detection, while an upstream, first region is excited by an antenna to condition or enhance a downstream measurement. The downstream coil (26) is tuned to detect heteronuclear resonances, while the upstream coil may be tuned for enhancement of the same or a different species. A cavity, in conjunction with the upstream coil (28a), allows populations and transfer coherence excitation between electrons and nuclei.



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## Tandem Coil NMR Probe

### *Background of the Invention*

Nuclear magnetic resonance (NMR) is a useful technique for determining molecular  
5 structure. Nuclei which have an odd atomic number or odd atomic mass possess magnetic  
moments and angular momenta. Such nuclei, when placed in an applied external magnetic  
field, will precess about the field with a frequency which depends upon the strength of the  
applied field  $B_0$  and the gyromagnetic ratio  $\gamma$  of the nucleus. Since every distinct nucleus has  
a unique gyromagnetic ratio, the precession frequency for each is different in a given  
10 magnetic field. For commonly used nuclei and conventional magnetic field strengths, the  
precession frequency is in the radio frequency (RF) range. If RF energy is applied at the  
appropriate frequency, the precessing nuclei will be excited and will consequently generate a  
signal which can be detected and processed to yield an NMR spectrum. These spectra  
contain a wealth of structural information and have made NMR spectroscopy one of the most  
15 versatile means for determination of the structure and conformation of molecules.

NMR is, however, a relatively insensitive spectroscopic technique, and typically  
requires large amounts of sample, compared to other spectroscopic methods, to yield useful  
results. This lack of sensitivity has restricted the use of NMR to those cases where  
considerable sample is available. Although it has been known for many years that NMR  
20 signals are enhanced in flowing liquids, the large sample volumes required to create a flow  
generally offer no benefits for fields such as protein analysis where the small quantities of  
sample available for analysis would require so much dilution as to negate any advantage  
gained by flow NMR.

There have been several reported applications of flow NMR in recent years. These  
25 have included liquid chromatographic detection (E. Bayer et al. *J. Chromatogr.*, **186**, 497  
(1979); F. Haw-James et al., *Anal. Chem.*, **52**, 1135 (1980)), measurement of flow rates and  
concentrations (A.I. Zhernovoi and G. D. Latyshev, "Nuclear Magnetic Resonance in a  
Flowing Liquid", Consultants Bureau, New York, (1965); M.A. Hemminga and P.A. De-  
Jager, *J. Magn. Reson.*, **37**, 1 (1980); H.S. Lew, U.S. Patent 4,901,018), detection of short-  
30 lived reaction intermediates (C.A. Fyfe, M. Cocivera, and S.W.H. Damji, *Acc. Chem. Res.*, **11**,  
277 (1978)), in vivo organ perfusion studies (N. Lavanchy et al. *Card. Adapt. Hemodyn.*  
*Overload, Train. Stress, Int. Erwin Riesch Symp.*, 228 (1983); K. Albert et al., *Z.*  
*Naturforsch. C: Biosci.*, **39C**, 859 (1984)), and dynamic electron-nuclear polarization (H.C.  
Dorn et al., *J. Magn. Reson.*, **79**, 404 (1988); R. Gitti et al. *J. Am. Chem. Soc.*, **110**, 2294  
35 (1988); S. Stevenson and H.C. Dorn, *Anal. Chem.*, **66**, 2993 (1994)). Still, many potential  
uses of flow NMR have not yet been made practical.

A further disadvantage of previously reported flow NMR systems has been the inability to exploit the many applications of NMR spectroscopy which make use of nuclear Overhauser enhancement or heteronuclear polarization transfer to enhance or otherwise clarify certain spectra. These phenomena have supported impressive advances in sensitivity and range for the analysis of non-flowing samples, but are not well adapted to flow NMR applications, because of difficulties associated with the evolving nature of these states and the isolated structures of NMR detection apparatus. These factors have generally limited implementation of nuclear spin enhancements to arcane experimental configurations of equipment, or to the measurement of static samples in conventional equipment.

Accordingly, it would be desirable to provide apparatus capable of implementing nuclear spin enhancements and performing observations with an increased range or greater sensitivity.

It would also be desirable to perform enhanced observations with heteronuclei, in flowing samples, or preferably both.

All of the above-cited references and publications, as well as the further publications specifically identified in the disclosure below are hereby incorporated herein by reference in their entirety for details of NMR machine construction and measurement techniques.

### *Summary of the Invention*

The present invention provides a novel probe for use in flow NMR spectroscopy. In one aspect of the invention, the flow probe includes a flow vessel which is positioned by a housing in a high magnetic field, and with tandem transmitter/receiver coils positioned sequentially along a fluid flow path through the vessel. This arrangement permits the use of nuclear Overhauser enhancement, and enhancement by coherence transfer techniques, such as spin-temperature labelling, of resonant signals by excitation of the sample nuclei in the flow volume at the upstream coil and detection at the flow volume of the downstream coil, while controlling difficult experimental factors such as residence time in the field.

In certain embodiments of the present invention, one or both of the upstream or downstream flow volumes includes a microwave cavity and a source of immobilized free radicals (IFR), which in this instance are configured to couple electron spin polarization to nuclear species, while retaining the closely-spaced high field NMR geometry and its functional advantages. The probe apparatus allows unique experimental protocols, for example, simultaneous excitation of a selected nucleus of the sample and also the unpaired electrons of the IFR material. This allows the implementation of electron-nuclear cross-polarization enhancement (also referred to as dynamic nuclear polarization or "DNP") of the

sample resonances. with potentially large increases in detection sensitivity of the sample resonances at the downstream coil.

5 In other embodiments of the invention. the flow path may include a reaction chamber situated so as to allow the initiation of a reaction and detection of the intermediates or products of that reaction at the downstream coil. With an upstream RF coil. certain of the reactants may be labeled or otherwise conditioned before reaction and detection.

10 In still other embodiments of the invention. a recirculation path. preferably with an integral pump. is provided to recycle the flowing fluid from a downstream point to a point upstream forming a closed loop flow path. In this fashion. the sample may be recirculated. allowing quite small volumes of sample and solvent to be used even for lengthy measurements taking hours or days.

15 In yet further embodiments of the invention. the probe may include a means for varying the size of a central part of the flow path to selectively control the flow time between the upstream and downstream coils. Such variation of the flow time adds an additional dimension in a multidimensional experiment such as reaction monitoring or the like.

20 In still further embodiments. the probe may be configured with two or more coils which are each tuned to separate nuclear Larmor frequencies with separate impedance matching and tuning means. for fine-tuning the resonance frequency and impedance of one or more of the coils. as may be necessary from one sample to the next. One or more of the coils. preferably the downstream coils. can be doubly-tuned. with one frequency corresponding to the Larmor frequency of deuterium or another suitable lock signal.

Novel protocols utilizing the probes include multidimensional and spin-temperature labeling procedures.

25

### *Brief Description of the Drawings*

Figure 1 depicts a generalized NMR apparatus.

Figure 2 shows an NMR probe according to the present invention.

Figure 3 illustrates RF coils disposed around a flow vessel in the subject NMR probe.

30 Figure 4 is a schematic representation of one embodiment of the subject NMR probe disposed in a magnet.

Figure 4A shows another embodiment similar to that of Figure 4.

Figures 5 shows an embodiment of the subject probe having a microwave cavity.

Figure 6 shows an embodiment of the subject probe with a reaction chamber and flow delay.

35 Figure 7 shows a  $^1\text{H}$  spectrum detected by a probe embodiment with toroidal coil.

Figure 8 shows detection sensitivity of the probe as a function of flow rate and flip angle.

Figure 9 shows  $^{13}\text{C}$  spectra of ethanol detected by the probe.

Figure 10 shows  $^{13}\text{C}$  spectra of  $\alpha$ -pinene detected by the probe.

Figure 11 shows another probe with multiple stages.

Figure 12 illustrates one embodiment of a flow delay element for use in probes of the  
5 invention.

Figures 13A and 13B illustrate another flow delay element.

Figure 14 illustrates a pulse sequence for a two-dimensional NMR measurement procedure.

Figure 15 illustrates a  $^1\text{H}$ -decoupled  $^{15}\text{N}$  spectrum of 80% N-methyl formamide in  $\text{CDCl}_3$ .

Sweep width 1850Hz. Spectrum from bottom to top: Statis spectra: flow rate of 6.0 mL/min  
10 with CPD decoupling, toroid inactive: flow rate of 6.0 mL/min with CPD on toroidal cell  
during recycle delay: DEPT spectrum under optimized conditions.

### *Detailed description of the Invention*

The uses of flow NMR reported in recent years have typically been restricted to those  
15 applications where large amounts of sample are available. Traditional means of enhancing  
sensitivity in static NMR experiments, such as nuclear Overhauser enhancement (NOE) or  
polarization transfer methods, have not been applicable to flow NMR studies. The ability to  
extend such techniques to flow NMR would be a valuable contribution toward increasing the  
versatility of flow NMR. The present invention addresses this problem by a novel NMR  
20 probe for use with conventional NMR spectrometers and magnets to perform NMR  
measurements on flowing fluids.

The term "flow NMR" as used herein refers to NMR spectroscopy of flowing fluids.  
Fluids contemplated for use in the invention include liquids, gases, supercritical fluids, and  
other fluids which are compatible with the probes of the present invention.

The lack of sensitivity of NMR techniques has been a severe hindrance to use of  
25 NMR methods, particularly in cases where the sample spectrum is complex and data  
acquisition times are long. The importance of increasing sensitivity can hardly be overstated.  
In modern research, the amount of sample available for spectroscopic measurement is  
frequently very small, and the difficulty of performing NMR analysis on minute quantities of  
30 sample often results in long data acquisition times if only small amounts are available, or  
else delays until more sample can be obtained. If the sample is unstable, long analysis times  
are simply not possible. The probe of the present invention increases sensitivity and  
decreases the time needed to accumulate sufficient data. The subject probe thereby makes the  
study of unstable compounds more facile. At many institutions, the amount of NMR  
35 instrument time available is limited and is carefully rationed; increased sensitivity helps to  
allocate this important resource more broadly. The probe also makes possible the use of  
lower concentrations of sample as well as smaller quantities of sample. The concentration of

the sample may be of crucial importance. For certain samples, for instance molecules which are capable of self-aggregation, changes in concentration may result in changes in the NMR spectrum, so that they can be detected, if at all, only when extremely dilute. Reaction products or intermediates may be both short-lived and present in minuscule quantities, and hence may be unobservable by NMR unless the sensitivity can be enhanced by an order of magnitude or more. The probe of the present invention therefore makes it possible to perform NMR measurements over a range of conditions which are not amenable to conventional systems.

Figure 1 shows the general features of an NMR apparatus which has a large electromagnet 1 generally maintained in a superconducting state within a housing filled with liquid helium and insulated by an outer shell filled with liquid nitrogen, a probe 2 which fits centrally within the housing to position a sample in a region of high and uniform magnetic fields, and a control and measurement system 3 which provides the necessary drive signals and receives the NMR signals for irradiation, detection and processing to derive a spectrum from a sample inserted in the apparatus and held by the probe. A conventional probe includes a vessel which holds the sample, an RF coil which surrounds the sample, and various tuning and impedance matching elements: generally speaking, trimmer capacitances and inductors which are connected to non-magnetic rods which serve as control elements for performing fine adjustments of the coil. These elements all connect to input-output terminals at the base of the probe, and the probe thus adapts the samples to reside in the inaccessible cryogenic center of the magnet and enables the necessary controls and measurement signals to be taken without interfering with the magnet.

Various details of construction and processing have become commonplace in NMR apparatus, and will be assumed without further discussion to be included in embodiments of the invention described below. Thus, the coils used in the subject probe may optionally be double tuned to the resonant frequencies of two different nuclear species, and the RF receiver coils in the subject probe are preferably configured to allow quadrature detection, or quadrature processing of detected fields. Furthermore, the probe of the present invention may be used with any magnet capable of providing a strong magnetic field, including permanent (iron) magnets, electromagnets, and superconducting magnets. When reference is made to a highly uniform field, it is understood that such uniformity may be achieved by providing a set of "shim" coils, which are energized to supplement and correct the basic static field. This is generally done under computer control in response to detection of the resonance frequency and linewidth of a simple species.

Figure 2 illustrates a basic embodiment of the probe 20 according to the present invention. As shown, probe 20 includes a vessel section 22 to be positioned in the magnet field, connectors 24a, 24b for placing the vessel in a flow segment, and coils 26 and 28



placed consecutively along the flow path through the vessel. Arrows F indicate the flow axis. In the discussion below, the terms "upstream" and "downstream" will be used without reference to whether the flow is actually up or down in the illustration of Figure 2: the actual flow direction may be selected depending on considerations of filling and bleeding of the vessel and other such factors. However, we shall routinely use the terms "upstream" and "downstream," generally identifying the downstream coil 26, illustrated in Figure 2, as the "primary" coil, meaning the one which performs a principal or ultimately intended nuclear magnetic resonance measurement, unless otherwise indicated or clear from the context. Thus, in discussions of measurements taken upon species flowing through the vessel, the RF signal induced in coil 26 will be subject to Fourier transform analysis, and the frequency spectra of the precessing nuclei will be generated from the signals therein.

Continuing with the description of Figure 2, each coil 26, 28 has associated therewith circuitry 27 and 29, respectively, for performing fine adjustments to the tuned frequency and impedance. Each coil is connected by leads, of which two, 30a and 30b, are shown, to shielded input-output junctions 33 in the base of the housing. In practice, the circuitry 27, 29 are located above or below the actual coil regions, so as not to affect field uniformity, but are illustrated adjacent to the flow region for expository clarity. Input-output junctions are set up to match an RF signal cable of standard impedance characteristics. In addition, a series of adjustment/control rods 34 of a non-magnetic material extend from outside the probe housing to the circuitry 27, 29 for performing fine adjustments of the capacitors or inductors and the coils. With this arrangement, the probe is set up to apply to and to receive RF signals from nuclei in two distinct regions along the flow path. The advantages of this construction will be discussed below in various examples. A more detailed description of the coils and flow vessel will be given with respect to Figure 3.

In Figure 3, the coils 26, 28 are shown disposed sequentially about the flow vessel 22. The coils are shown as saddle coils, but it will be understood by one skilled in the art that other known coil configurations for applying RF fields may be used. The flow vessel 22 extends through a conditioning zone 40, a transition zone 45 and a primary detection zone 50. In the present invention, various forms of conditioning or measurement are contemplated in zone 40 upstream of the primary coil as well as, in certain embodiments, a reentrant flow path for effecting enhanced measurements.

Figure 4 shows the probe of Figure 3 disposed within the magnet. A pump 64 (such as a pneumatically driven pump) and a recirculation loop 62, both shown in phantom, are preferably provided when analyzing small amounts of sample. The RF coils 26 and 28 are transmitter/receiver coils, and each coil preferably further includes an additional, generally one or more concentric coil (not shown in the figure) surrounding the same flow volume, as is known in the art, for providing a field/frequency lock (e.g. a deuterium lock) or a nuclear

magnetic resonant frequency different from that of the respective accompanying coil, as for example in decoupling and heteronuclear correlation experiments. In certain preferred embodiments, one or more of the coils can be tuned to more than one frequency, e.g., such as tuned to both  $^1\text{H}$  and  $^2\text{D}$ . Likewise, it will be understood that the pump mechanism can be  
5 (optionally) partially disposed or completely disposed outside of the probe housing, particularly where it is desirable to use an induction motor or the like.

The terms "coil" and "RF coil" are recognized in the art and are used interchangeably herein. The term "RF coil" refers to an electrical conductor, e.g., wire or tape with non-zero inductance, which is often coiled shaped and which can be used to transmit and receive  
10 radiofrequency signals. RF coils are preferably made of copper or other electrically conductive but non-ferromagnetic materials. The RF field generated by a coil is preferably orthogonal to the strong applied magnetic field  $B_0$ . Exemplary RF coil configurations which may be useful in the present invention are solenoidal, saddle, Helmholtz, modified Helmholtz, Golay, and toroidal coils. Preferred embodiments employ coils providing  
15 uniform fields.

In the discussion which follows, the invention will be applied to difficult measurement problems which have arisen in modern NMR equipment having very high magnetic fields. In general the degree of resolution and sensitivity obtainable depend on the field strength as well as on the homogeneity achievable in the field. Typical high-  
20 performance machines are characterized by proton resonant frequencies of 400 MHz to 750 MHz, corresponding to magnetic fields of 9.4 to about 18 Tesla; however, the principles of the present invention are not limited to such machines. The terms "high field" and "high magnetic field" as used herein are intended to refer to a magnetic field with a field strength of about at least 0.5 Tesla (T), preferably in the range of about at least 1T to about 24T, and  
25 more preferably about at least 2.1T to about 24T.

### Example 1 (Prototype Embodiment)

Figure 4A schematically shows the design of a sequential-coil flow-probe in one prototype embodiment. The  $^{13}\text{C}$  transmit/receive downstream coil 26 surrounded a 5 mm o.d. Pyrex vessel with a volume of 0.19 ml and was surrounded by a  $^1\text{H}$  decoupling coil (not  
30 shown) of conventional type. The Pyrex cell was joined to Teflon tubing with Teflon tape.

A prepolarization chamber which is large compared to the detection coil helps the sample attain Boltzmann equilibrium and NOE enhancement before detection. Two types of prepolarization chambers have been constructed, giving comparable enhancements. One was  
35 built of 10mm Pyrex tubing inside a conventional saddle-shaped Helmholtz  $^1\text{H}$  coil. The other, depicted as a toroidal coil 28a was used as the upstream coil to enclose a large volume.

with a geometry that allowed it to be mounted close to the homogenous region of the magnet, and has a large filling factor with good Q (T.E. Glass and H.C. Dorn, *J. Magn. Reson.* **51**, 527 (1983)). The toroid 28a was made by winding six turns of commercially available coil wire (silver-plated copper) around a Teflon spool. The sense of winding was reversed after  
5 the first three turns to reduce the inductance of the coil and facilitate tuning to high frequencies (S.B.W. Roeder and E. Fukushima, *J. Magn. Reson.* **59**, 307 (1984)). The toroidal coil 28a was insulated with Teflon tape to prevent arcing. Around the spool inside the toroid, approximately 1.3 m of Teflon tubing (1/16 inch inner diameter) was wound up, holding 1.6 ml of sample. This design avoids excessive sample mixing during the passage  
10 through the toroid. An alternative design employing a double-saddle Helmholtz coil around a 10 mm diameter Pyrex cell has also been tested, and provided comparable results.

The tuning circuit for the upstream coil 28a, including three non-magnetic high voltage Johanson variable piston capacitors (0.8 - 10pF), was mounted above the transmit/receive coil of a commercially available BRUKER <sup>13</sup>C probe which was suitably  
15 modified to receive the additional coil and tuning circuitry, and good grounding was achieved by a solid connection to the housing of the probe. Connection to the circuit was made with a non-magnetic 50 ohm coaxial cable lowered through the magnet bore and fastened to a Sealelectro SMB jack on top of the probe. The center of the toroid coil 28a lies 3.2 cm above the primary transmit/receive coil 26. Figure 7 shows a <sup>1</sup>H "spectrum" recorded with the  
20 toroidal coil. The signal has a width of almost 20 KHz and is shifted by almost 20 KHz, relative to that obtained at the decoupler coil of the primary coil 26 below. The sharp signals visible in the center of the spectrum come from the decoupler coil which resonates with the toroid.

The low power output of the proton decoupler of a BRUKER AM 400 NMR  
25 instrument was further amplified by a 2W linear amplifier to apply a composite pulse or continuous wave (CW) RF signal to the toroidal coil. Series crossed diodes and a 400MHz bandpass filter on the amplifier output were employed for reducing noise in both observe and deuterium lock channels. The 90° flip angle of the toroid was approximately 140 ms.

A Millipore peristaltic pump employing 5mm o.d. Masterflex Tygon tubing was used  
30 for sample recirculation.

#### Determination of Optimal Parameters

To show the individual contributions of flow and NOE enhancement, the acquisition parameters for each experiment were optimized within the hardware limitations discussed above. Static experiments were optimized according to well-known principles (R.R. Ernst et al., *Principles of Magnetic Resonance in One and Two Dimensions*, Clarendon Press, Oxford,  
35 1987). The acquisition time  $t_{aq}$  is determined by the necessary sweep width and digital

resolution (in practice, acquiring for 2 to 3 times  $T_2^*$ , the effective transverse-relaxation time). Keeping pulse repetition time  $T_{rep}$  less than  $T_1$ , choosing pulse angle  $\beta$  equal to the "Ernst angle" (Ernst et al., *supra*), and applying matched filters before processing leads to optimal  $(S/N)_1$ . In flow experiments,  $T_{rep}$  must be suited to the flow rate so that  $T_{rep}$  equals  $\tau_{det}$ , the sample lifetime in the detection cell ( $\tau_{det} = \text{volume}_{det}/\text{flow rate}$ ). Our simulations of steady-state magnetization are based on a classical vector model with plug flow enable determination of relative sensitivities, optimal flow rates, pulse angles  $\beta$ , and  $T_{rep}$ . Figure 8 calculated for parameters typical for ethanol shows that  $(S/N)_1$  reaches a maximum for a specific flow rate and pulse angle. For  $T_1$  and  $T_2^*$  values of 3.5 and 0.1 s. and a  $t_{aq}$  of 0.25 s. our simulations reveal optimal  $(S/N)_1$  for a flow rate of 19.3 ml/min, a pulse angle of  $90^\circ$  and a  $T_{rep}$  of 0.59 s. Our studies show that the correct flip angle for flow NMR is always  $90^\circ$  when  $T_{rep}$  equals  $\tau_{det}$ , and the flow rate is optimal when  $\tau_{pre}$ , the sample lifetime in the prepolarization volume, is between 1.1 and 1.8  $T_1$ .

### Results and Discussion

Some proton-coupled  $^{13}\text{C}$  spectra of 90 % ethanol in  $\text{D}_2\text{O}$  containing a small amount of acetic acid (for catalyzing proton exchange) are depicted in Figures 9A-9D. To compare sensitivities of spectra, we determined optimal parameters for each individual spectrum, and used always the same total acquisition time. For a static sample (zero flow rate), the best response is achieved employing a flip angle of  $\beta = \arccos(e^{-T_{rep}/T_1})$  (R.R. Ernst and W.A. Anderson, *Rev. Sci. Instrum.* **37**, 93 (1966)). The longitudinal relaxation times of the methyl and the methylene group of ethanol are 5.5 s and 3.5 s, respectively. The spectra recorded with a repetition time of 2.2 s and a flip angle of  $60^\circ$  are shown in Figure 9A. All spectra have been processed using a matched filter (exponential line broadening, 3Hz).

To obtain full premagnetization and optimal flow signal enhancement, a flow rate of 25 ml/min was chosen. The optimal repetition time for a 0.19 ml detection cell is 0.45 s. To obtain a satisfactory digital resolution, 4K of data points were recorded at a sweep width of 8065 Hz. This set of parameters leads to an acquisition time of  $t_{aq} = 0.25$  s. Twelve scans were coadded, preceded (as in all spectra reported here) by eight dummy scans). For these parameters, the duty cycle of the toroidal coil 28 is only 44%, because the composite pulse decoupling (CPD) sequence cannot be applied to the toroid during acquisition. This is a consequence of the fact that the decoupler coil resonates with the toroid 28a, which causes partial decoupling during acquisition. A circuit that untunes the decoupler coil during acquisition would most likely remove this problem, and this would be provided in a further embodiment by a synchronous switching circuit that connects an additional inductive or capacitive circuit element to the decoupler coil to shift its resonance and damp oscillations during acquisition. For the present experiments, a compromise that provided a better duty cycle (89%) for the toroidal coil 28a was chosen with a flow rate of 6 ml/min and a repetition

time of 2.2 s. This set of parameters allows the sample to experience only one 90° pulse in the detection cell; however the prepolarization and NOE buildup are both reduced. Figure 9B shows the spectrum at a flow rate of 6 ml/min without any RF pulses applied to the toroid 28a, in which it is seen that the S/N ratio is approximately twice that of the static spectrum.

5 The NOE enhanced spectrum induced by a CPD sequence applied through the toroid 28a (gated off during acquisition) is shown in Figure 9C. Despite the inhomogeneity of the magnetic field around the toroid and the non-optimal flow rate, a NOE enhancement factor of 2.3 is achieved. DEPT (Distortionless Enhancement of Polarization Transfer) spectra (Figure 9D) give an even higher enhancement; however it suffers from the fact that the signals of

10 quarternary carbons completely disappear from the spectrum. The DEPT spectrum has been recorded with a shorter repetition time and an increased number of scans (16 scans, but the same total acquisition time), in view of its dependence on the relatively shorter relaxation times of the protons.

Some <sup>13</sup>C spectra of α-(+)-pinene (40% in CDCl<sub>3</sub>) are shown in Figure 10. The α-

15 pinene has been distilled prior to the acquisition of the spectra. <sup>13</sup>C longitudinal relaxation times at 100.26 MHz ranged from 17 s (C6), 14 s (C2), 8.5 s (C1), 7 s (C3, C5) to ~5s (C4, C7, C8, C9, C10). Acquisition conditions were optimized for the longest T<sub>1</sub> and were as follows. Spectrum 10A was recorded with no flow, repetition time 3.2 sec., 48 scans CPD decoupling during acquisition with the toroidal coil 28 inactive. Spectrum 10B was recorded

20 with a flow rate of 1.9 ml/min., repetition time of 3.2 seconds, CPD or CW on the toroidal coil during recycle delay, and CPD decoupling on the downstream decoupler coil during acquisition with the toroid inactive. Spectrum 10C was taken under the optimized conditions, at a flow rate of 1.9 ml/min, repetition time of 1.8 sec., 64 scans. The flow rate was adjusted by trial and error, and showed that a flow rate of 1.9 ml/min gave a much better response than

25 the calculated flow rate of 4.5 ml/min. This is a consequence of the higher viscosity of α-pinene, which causes a pronounced laminar flow effect. Thus, part of the sample passes the prepolarization coil before the full magnetization is built up. This cannot be compensated by another fraction of the sample that stays longer than required in the prepolarization volume, because the maximum longitudinal magnetization is limited by the strength of the magnetic

30 field. However, the higher T<sub>1</sub> values can in principle be offset by a larger pre-polarization volume.

All pinene spectra are recorded with the same overall acquisition time of 115.4 s. Composite pulse decoupling was achieved by switching between the low power decoupler for the toroid and the high power decoupler for the decoupler coil, whose carrier frequencies

35 differed by 20 KHz. In initial experiments, simultaneous excitation of both coils was not possible, because only one decoupler was available. A higher number of data points (16 K) and, consequently, a longer acquisition time, allowed a duty cycle of approximately 80% for the toroidal coil to be achieved.

The overall enhancement factors obtained for  $\alpha$ -pinene are between 1.5 and 2 for a flow rate of 1.9 ml/s using the toroidal coil for NOE enhancement, and are very similar for flow DEPT. The quaternary carbon signals (C2, C6) completely disappear in the DEPT spectrum (Figure 10C).

5  $^1\text{H}$ -decoupled  $^{15}\text{N}$  spectra of 80 vol % *N*-methylformamide ( $T_1 = 11.8$  s) in  $\text{CDCl}_3$  depicted in Figure 15 were recorded with the same probe with the  $^{13}\text{C}$  coil retuned to  $^{15}\text{N}$ . The resonances of all spectra in Figure 15 are phase inverted because of the negative NOE of  $^{15}\text{N}$ , even for a  $^1\text{H}$ -decoupler duty cycle ( $T_{\text{rep}}/t_{\text{aq}}$ ) as low as 8% in the static spectrum (Figure 15, bottom spectra). The flowing of sample in Figure 15 results in reduced NOE because of  
10 lower  $\tau_{\text{det}}$ , which tends to cancel the signal despite the increased decoupler duty cycle (32%). Strong NOE enhancement is shown in Figure 15 (2nd spectra from top) with the decoupler time-shared between the toroidal and decoupler coils (toroidal duty cycle = 68%). Our simulations for this case predict an overall enhancement (flow + NOE) of -8.8. The  $^{15}\text{N}$  DEPT spectrum (see Figure 15, top spectra) with a  $T_{\text{rep}}$  reduced to accommodate the smaller  
15  $T_1$  of the directly bonded amide proton, gave approximately 30% higher  $(S/N)_1$  than the spectrum recorded with CPD on toroidal cell during recycle delay.

It should not be very surprising that, given enough time, the NOE can be obtained for flowing samples, or that enhancement can be accomplished with relatively small amounts of RF power. The amounts of NOE enhancement are orders of magnitude less than that  
20 achieved in flow DNP (S. Stevenson and H.C. Dorn, *Anal. Chem.* 66: 2293(1994)), but for many applications, e.g., metabolic studies and LC detection, it should be well worth the modest addition in hardware to recover this normally wasted sensitivity.

What is perhaps more significant is a host of new possibilities suggested by the tandem flow-cell arrangement for analytical FT NMR, including the study of reaction  
25 mechanisms, short-lived intermediates, and pathways by "spin-temperature labeling." For example, a reactant resonance in an upstream cell could be inverted by a selective pulse prior to rapid mixing with a chemical reagent and detection in a cell downstream. This could provide an alternative to expensive and time-consuming isotopic labeling, for example, in the study of molecular rearrangements. Such applications would require magnetic homogeneity  
30 at the upstream cell comparable to that existing downstream, possibly demanding a special magnet and/or dual shim systems. The NOE enhancement method describe here, however, requires little magnetic homogeneity upstream and should be applicable to all heteronuclei where  $^1\text{H}$  dipolar interactions contribute to relaxation.

Returning now to a description of the apparatus, the invention also contemplates a  
35 probe including a flow chamber having an NMR detection region, and further including a microwave cavity located along the flow path for polarization conditioning or signal enhancement of a nuclear type of interest. One such device is shown in Figure 5, wherein a

microwave cavity 52 is provided at the upstream coil 28, and an environment of immobilized free radicals 54 in the flow region 40. Thus the microwave cavity 52 is situated in the magnetic field, and the material passing along the flow path is simultaneously subjected to the stationary magnetic field  $B_0$  and to interaction with the captive electrons of the radicals immobilized in the microwave cavity, as well as the RF fields  $B_1$  from the upstream coil. A representative example of operation of this probe follows.

### Example 2

In an exemplary protocol, a sample is introduced at an upstream flow position and flows into the microwave cavity 52 to interact with a source of immobilized free radicals (IFR) on a solid support. Suitable sources of IFR are known, and may be, for example, silica-phase immobilized nitroxide radicals (R. Gitti et al., *J. Am. Chem. Soc.* 110, 2294 (1988)). Other free radicals such as phenoxide have been reported, and the choice of species may depend in part on considerations of compatibility with the sample as well as empirical selection factors such as efficiency and ease of preparation. The microwave cavity is a conductive walled chamber which resonates at the microwave frequency, discussed further below.

Briefly, the following protocol seeks to enhance the polarization (hence detectable signal) of a with a small gyromagnetic ration nucleus by introducing the desired spin into an abundant electron species and coupling that spin into the nucleus. The technique used is analogous to the classical technique of cross-polarization-magic angle spinning (CP-MAS) used to couple spin polarization between pairs of nuclei in solids, to take advantage of the greater excited state population available in one species. This polarization coupling requires that the target nucleus be conditioned to change spin upon receiving the quantum of energy available from a spin transition in the abundant donor nucleus, generally a proton. Because the electron gyromagnetic ratio is much greater than that of e.g. carbon ( $\gamma_e/\gamma_C = 2628$ ), and its precession frequency correspondingly great, conventional wisdom would not normally suggest NMR signal enhancement by electron-nuclear spin interactions. This technique is known in the art as Dynamic Nuclear Polarization.

The probe of Figure 5 provides necessary elements for controlling such a coupling interaction between electrons and nuclei. As described further below, this is done using the upstream cavity and coil by spin-locking both the electrons and the nuclei of interest away from the static magnetic field  $B_0$  and arranging that the strength of the orthogonal magnetic fields induced by the two irradiation sources satisfies the Hartman-Hahn condition on gyromagnetic ratios for spin coupling of two particles.

The nuclear type of interest is excited by spin-locking according to established methods (for details of suitable spin-locking procedures, see e.g. G.A. Morris and R. Freeman, *J. Am. Chem. Soc.* 101, 760 (1979)), so that the spin is locked in the magnetic field  $B_1$  of the RF coil, which in this case is the X-Y plane, orthogonal to  $B_0$ . In an analogous fashion, the microwave transmitter establishes a standing microwave field with a magnetic component rotating in the X-Y plane, which interacts with the spin of the unpaired electrons of the immobilized free radical phase so that these electrons are also spin-locked and in the desired orientation. As with the corresponding nuclear-nuclear cross-polarization coupling techniques, the two fields are preferably locked in phase relation. For lower static magnetic fields  $B_0$  (hence longer microwave electron spin resonance wavelengths), this may be accomplished with a doubly resonant cavity/coil configuration. For higher microwave frequencies necessary in very strong static magnetic fields, this may be achieved by suitable frequency dividing and phase feedback controls, possibly with special mixing or doubling resonators operative in the short microwave region. As with nuclear cross-polarization, the amplitudes of the two locked fields are adjusted to satisfy the Hartman-Hahn ratio. Preferably, the electrons are spin-locked before the nuclei, though electron spin-locking can be accomplished simultaneously or after spin-locking the nuclei, and the RF field then applied to the nuclei to bring both spin systems into alignment. The polarization of the excited free radicals is then transferred to the spin-locked nucleus of interest as the fluid flows over the solid support. Since electrons have a much greater energy difference between up and down spins, this leads to a greatly enhanced population of the coupled spin polarization in the flowing nuclei. When the fluid flows downstream, this increased polarization is detected at the second coil as a greatly enhanced signal in the spectrum of the nuclei. The construction of the upstream coil in combination with the microwave cavity and immobilized free radicals thus provides a structure for exciting electron spin and coupling this spin to a heteronucleus. This mechanism may in theory produce signal enhancements of up to 2628 for  $^{13}\text{C}$  and 6570 for  $^{15}\text{N}$ . Such enhancements would correspond to reductions in data acquisition time for a two hour experiment to seconds.

### Example 3

The embodiment of the invention shown in Figure 4 may be used for the following protocol. The active regions of the first and second flow volumes 40b and 50a, and the first and second RF coils 28 and 26 are each connected to RF transmitter(s) and receiver(s) and tuned to the same nuclear resonant frequency. No recirculation loop is needed for this experiment. A sample is introduced into the flow vessel and flows to the region of the first coil 28. A soft pulse sequence or other specially calculated selective  $^{13}\text{C}$  pulse or RF signal is applied to the first coil 28 to excite (or, in a related protocol, to invert) individual  $^{13}\text{C}$



resonances, e.g., the resonance of a particular carbon atom in a first reactant molecule, or a selected set of resonances. Such selective pulses are well known in the art and have been described for static samples (see e.g. G.A. Morris and R. Freeman, *J. Magn. Reson.* **29**, 433 (1978)). The sample then flows to a reactor located between the coils, where a second  
5 reactant is introduced. This initiates a set of chemical changes at a precise time and exact location along the flow path, which may occur instantaneously, but most preferably for the full practice of this method the reaction evolves over time according to a defined reaction mechanism or set of paths. Since the fluid is flowing the species present at a given point for an evolving reaction varies with position along the flow path. Thus, after a delay, the  
10 products (or chemical intermediates) of the reaction are detected downstream. This is done with a standard  $^{13}\text{C}$  read pulse at the second coil 26. The spectrum thus obtained will have a perturbed line, that reveals which carbon atom of the product (or chemical intermediate) molecule corresponds to the previously perturbed atom in the defined position which was spin-temperature labeled in the reactant molecule upstream, thus shedding light on the  
15 reaction pathways. The process may be repeated to trace the outcome of reaction for all atoms in the first reactant molecule. This experiment largely obviates the need for difficult and expensive specific isotopic labeling of the first reactant molecule in the study of chemical reactions. Figure 6 is a schematic view of the probe of Figure 4 showing the reaction chamber 60 located in the position 45 of the flow vessel intermediate the two coils. For the  
20 practice of this method, Figure 6 also shows a flow delay element 70 and a reactant injector 65 connected to the reactor 60 in the flow path.

The embodiment shown in Figure 6 is used for the study of a variety of chemical reactions, such as molecular rearrangements in a similar manner. The first reactant molecule passes through the first flow volume where it is excited by the first coil as described above.  
25 It then enters the reactor. Illustratively, the reactor need not introduce another chemical reactant, but may simply initiate a rearrangement or fragmentation reaction, e.g. by cooling, irradiation with ultraviolet light, or contact with an immobilized catalyst or enzyme in the reactor. After flowing through a certain length as before, the intermediates or products of the reaction are detected by the second RF coil, thus revealing information about the course of  
30 the reaction or rearrangement. The delay element provides a definite and preferably variable delay path to precisely control the time instant following initiation at which downstream detection occurs. Delay element 70 may for example consist of a telescoping U-tube in the flow path, which provides a variable path-length of fixed cross-section connecting the flow paths. The use of such a delay element results in a very dependable and repeatable  
35 mechanism for time sampling of the reactant products, since the sampling delay can be controlled without changing the transit times through either the first or the second coil.

The embodiment shown in Figure 6 is also used in yet other novel protocols. In one such technique, the first coil and the second coil are each used as both transmitters and

receivers. The first coil obtains an NMR spectrum of the first reactant according to standard protocols. The reactant then flows to the reactor where a reaction is initiated (e.g. by heat, light or the like, as described above). After flowing through the delay volume, the spectrum of newly evolving intermediates or products is detected by the second coil. A difference spectrum is then generated by subtracting the first spectrum from the second spectrum. This has the effect of canceling solvent peaks and other peaks common to both spectra, e.g., to filter spin-temperature labeled peaks. This procedure has the advantage of reducing receiver dynamic range problems associated with large solvent peaks. Provided the volumes within the coils and the fields are sufficiently well matched, the differencing operation may be performed on intermediate or transformed data sets rather than fully analyzed spectra, greatly reducing computational load and/or storage requirements. For such operation, rather than the dissimilar tubing shown for the upper flow chamber shown for Figure 6, a flow chamber identical to the lower one would be provided. To facilitate such configurations, flow chambers, reaction vessels and delay portions and the like may be provided in modular, snap-together embodiments.

The invention further contemplates a probe as shown in Figure 11 including a flow chamber having two NMR detection regions, and further including a premagnetization volume and a reaction chamber, both upstream of both coils, and a delay path intermediate the two coils. In Figure 11 a premagnetization volume 80 is provided in the flow region 40, and a reaction chamber 60 is included in the flow volume 40 upstream of the first coil. The probe also includes a delay element 70 and a second detection coil. An exemplary protocol for operation of this probe follows in Example 4, after a general discussion of the additional elements of the premagnetization volume and reaction chamber.

For many measurements, the attainment of maximum sensitivity requires the sample nuclei to be pre-equilibrated in a region of high magnetic field to establish the Boltzman distribution of spin states. It is therefore important to provide the sample with sufficient residence time in the high magnetic field before NMR measurements begin. The premagnetization volume is preferably made of a nonmagnetic material, for example Teflon or Pyrex. One premagnetization volume suitable for allowing such residence time is the upper chamber already shown in the region of the upper coil in Figure 6, which may perform this function when operation of the upper coil is not required. In that embodiment, a length of Teflon tubing 75 is wound around a spool. This construction allows one to select a residence time in the field by selecting an appropriate size and length of tubing, while arranging that the fluid arrives as a bolus with substantially uniform residence characteristics. Another exemplary embodiment employs a Pyrex flow vessel having a large diameter relative to the diameter of the flow vessel immediately upstream to augment residence time. In still another embodiment, the flow vessel comprises an outer tubular member and an inner tubular member, fitted together with a fluid-tight seal, sharing an axis and having a length along that

axis. One tubular member is movable relative to the other, and the volume within the vessel may be varied by sliding the movable member along the shared axis, much like the telescoping delay path configurations described above. This construction capitalizes on the increased volume of the delay section, without any changes in coil or magnet shims, but does not necessarily utilize the available space in the high field very efficiently.

In the context of the present invention, the illustrated reaction chamber is a small flow volume of the flow vessel, preferably constructed of a nonmagnetic material, arranged to produce turbulent mixing or intimate and complete contact or treatment of the sample as it passes through the chamber. In certain embodiments, the reaction chamber will have fittings by which flow tubing for the sample and a reagent or reagents may be connected. The reaction chamber may also be constructed with a temperature-control arrangement such as a heater. In certain embodiments, the reaction chamber may be equipped to initiate or facilitate or complete a process by irradiation, by using, for example, ultraviolet light or a laser light source. One reaction chamber suitable for use in the probe of the present invention has been described in U.S. Patent 5,198,766, of inventors M. Spraul and M. Hofmann.

While not shown in Figure 11, a reentrant flow path such as shown in phantom in Figure 4, may be useful to provide recirculation of the fluid flow from a downstream region to an upstream region, thus forming a closed loop, allowing multiple passes through a region, before or during a measurement. Fluid may be made to flow in the loop 62 by a pump which may be pneumatically powered, preferably by an air source such as is commonly present in NMR machines for spinning sample turbines in the magnet assemblies. The pump is made of nonmagnetic materials, and is preferably a peristaltic or impellor pump.

#### Example 4

In a representative protocol for the probe of Figure 11, the fields and chambers in the active regions of the first and second flow volumes A and B are well matched, and the coils 26 and 28 are also well matched. A sample is introduced at a position upstream of the flow region and flows into the premagnetization volume 80 in the static magnetic field. The volume of chamber 80 is sufficiently large that the residence time of a flowing bolus of fluid is long compared to  $T_1$ , insuring adequate premagnetization and thus maximum sensitivity of detection. The sample then flows to the reaction chamber 60, where a process is initiated or a reactant is introduced. This initiates a set of chemical changes which evolve over a period of time. The sample flows on, to the first detection region A, where a first NMR spectrum is obtained by coil 28 by a conventional method. The sample then flows to the delay element 70, where the reaction is allowed to further evolve for a defined period. The sample then flows to the second detection region B, where a second NMR spectrum is obtained, using

signals on coil 26. Thus, the same measurement conditions are applied to a single sample which has evolved as it flows between coils. In a basic embodiment of this aspect of the invention, the spectrum obtained at the first coil 28 is subtracted from the spectrum obtained at the second coil 26. The resulting difference spectrum reveals only those components of the spectrum (lines or line amplitudes) which have changed during the reaction course. The difference procedure also effectively cancels the solvent and other large constant peaks, such as those due to excess reagents, which can be a significant factor in reducing dynamic range difficulties when the desired resonances are weak. Further, the time delay introduced by the delay element 70 may be changed in a systematic manner, allowing the study of the reaction course over time. This time delay may be used as one dimension of a two-dimensional experiment, as described elsewhere. Further, provided the two coils, chambers and surrounding field conditions have been matched sufficiently closely, the difference procedure may be applied to intermediate blocks of data or data transforms, thus greatly reducing the volume of data processing or storage required to obtain useful spectra.

Returning to the description of the time delay path element 70, it will be understood that a variety of structures can be used to vary the length of time between when the volume of the fluid is irradiated in the upstream coil and when it is finally presented within the downstream coil. Figure 12, for example, illustrates an embodiment of a delay element 70' which provides a set of telescoping U-tubes 100 which can be disposed in the flow path as part of the flow vessel 22. The length of the path between inlet and outlet 102A, 102B of the nested U-tubes 100, with consideration of the flow velocity, defines the delay time provided by delay element 70'. Accordingly, as illustrated in Figure 12, extension (or compression) of the telescopic portion 104 between each of the U-tubes 100 varies the length of the flow path through the delay element 70' and, when the flow velocity is constant, permits incremental increases (or decreases) in the time it takes for a bolus of sample conditioned in the upstream coil to reach a downstream position for further mixing and detection.

Figure 13A and 13B illustrate an embodiment of a delay element 70" which provide a set of nested, telescoping U-tubes 100' which permit radial expansion of the delay element in the intercoil region, thereby making use of space within the probe without reconfiguring the coils or magnets. In one embodiment of the delay element of Figure 13A, the tube support members 106 are moved relative to the axis of the telescopic portion 104 by an air or fluid actuated mechanism.

In yet another exemplary embodiment, delay element 70 can be provided in a plurality of valves for switching tubing of different lengths in and out of the intercoil flow path of fluid vessel 22. For instance, in one embodiment a carousel can be supplied which comprises individual coils of different lengths of tubing. The carousel is rotated to couple an inlet and outlet of a given tubing as part of the fluid vessel 22 within the intercoil space by a fluid-tight

coupling. In other embodiments, tubing of various length can be connected and provided in the flow path of fluid vessel 22 by the use of selectively-actuated pinch-valves.

There are a number of different reasons Applicants provide a time-delay, e.g., by variable volume or flow path length, in the flow path of the intercoil space for both one-dimensional and multi-dimensional NMR experiments. As indicated in one example above, by balancing the flow rate with the duty cycle on the pulse generator (e.g. periodicity of nutation pulses) and other acquisition parameters one can optimize sensitivity of a flow measurement. Moreover, in the instance of those embodiments which provide a reaction chamber, the inclusion of time-delay element which can be varied by the operator, as shown in Figure 6, can permit the acquisition of spectra at incremental time points after a reaction has been initiated. For instance, the dimensions of the time-delay element can provide a time-delay in the range of milliseconds to resolve the formation or fate of short-lived intermediates..

In another embodiment, the time delay feature of the subject probe can be used to generate two-dimensional (or multi-dimensional) NMR spectra. An important strength of modern NMR spectroscopy is the ability to drive the dynamics of the spin system through a series of coherence transformations such that the resultant observable magnetization reflects desired combinations of evolutions and interactions. Coherence transformations can be used not only for transferring magnetization from one spin to another (such as described in detail above), but also for "filtering" purposes to select only those spin systems with a given property. Coherence transformations can be used, for example, in a two-dimensional (2D) NMR experiment to display correlations between coupled spins. This can be accomplished in the subject probe by exciting coherences with a preparation pulse sequence at the upstream coil, then allowing them to evolve for a variable time  $t_1$  (Figure 14) which is the time it takes the conditioned fluid to flow from the upstream to the downstream coil. After the evolution period, a mixing pulse sequence is applied to the system by application of one or more nutation pulses at the downstream coil, followed by observation at the downstream coil of the (-1)-quantum coherences during the period  $t_2$ . The resulting FID signal is processed in a manner similar to the one-dimensional experiment by taking the Fourier transformation of the signal. However, a second time dimension is introduced by repeating the experiment while systematically varying the length of the evolution time period  $t_1$ , e.g. by varying the time it takes for a fluid sample to move from the upstream coil to the downstream coil. The resulting set of FIDs are then subject to a second Fourier transformation over the varying time of the evolution period. The two frequency spectra can be plotted along the horizontal and vertical coordinates of a graph to produce a two-dimensional plot. This plot has "peaks" which represent coherence transformations and, therefore, is useful in studying, for example, molecular structures as it can contain information pertinent to the three-dimensional geometry of the molecule.

Coherence transformations can be used, for example, in homonuclear two-dimensional (2D) NMR experiment to display correlations between coupled protein spin systems. A typical proton homonuclear 2D experiment involves the interaction of each proton ( $^1\text{H}$  nucleus) with all of its coupling partners, such as through-space or through-bond couplings. In an exemplary measurement protocol, the protons are excited during the preparation time period by an application of an RF nutation pulse at the proton resonant frequency through the upstream coil. The resulting coherence evolves under influence of the chemical shift during the  $t_1$  evolution time period as the conditioned fluid flows through the fluid vessel in the intercoil space. The coherence is then transferred between coupled protons during the mixing period by applying a second RF pulse to the protons via the downstream coil. Finally, during a detection period, the FID is sampled at the downstream coil. The resulting FID signal is modulated not only by the chemical shift evolution of the protons during the detection period but also by the spin evolution period of coupled protons. Such experiments include CORrelation SpectroscopY (COSY) and NOE Spectroscopy (NOESY) experiments. For instance, in COSY experiments and other through-bond sensitive experiments, correlation of (-1)-coherence through J-coupling can be used to establish that the coherences belong to the same coupling network. Other homonuclear experiments, as well as the appropriate configuration of the subject probe, will be apparent to those skilled in the art in light of the present disclosure.

Similar multi-dimensional experiments can be performed in heteronuclear embodiments of the subject probe, as for example, 2-dimensional NMR experiments specifically designed for the detection of proton-coupled  $^{15}\text{N}$  or  $^{13}\text{C}$  chemical shifts. For instance, polarization transfer experiments based on an INEPT or reverse-INEPT pulse sequence between the upstream and downstream coils, such as a reverse Heteronuclear Multiple Quantum Coherence (HMQC) 2-D experiment, can be used to make resonance assignments.

The invention being thus disclosed and the operation of representative embodiments described, variations and modification of the probe apparatus, and a wide range of homo- and heteronuclear measurement techniques which can be performed with one or more different embodiments of the subject probe will be apparent to those skilled in the art in light of the present disclosure, and all such variations, modifications and embodiments are considered to be within the scope of the present invention, as set forth in the claims appended hereto.

What is claimed is:

1. A probe for holding a sample of material in a test region of high magnetic field and uniformity in an NMR apparatus, such probe comprising:

- 5 a housing for positioning a sample-holding body in a test region and a body secured to said housing, said body having a flow chamber with an opening for introducing fluid to flow along a flow path through said test region, the flow chamber comprising in flow sequence
- 10 a first flow volume disposed in a first region  
a second flow volume disposed in a second region downstream of and adjacent to the first flow volume  
so that flow through said first and second regions experiences the high field of said test region:
- 15 a first coil disposed in said first region and tunable to a first nuclear resonant frequency:  
a second coil disposed in said second region and tunable to a second nuclear resonant frequency:  
whereby excitation of said first coil when a sample flows through said flow chamber enhances nuclear resonance detected by said second coil.

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2. A probe according to claim 1, further comprising flow recirculation means in said body for providing a return flow path from said second to said first flow volume.

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3. A probe according to claim 1, further comprising a microwave cavity in said body located in said first region.

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4. A probe according to claim 3, wherein the first flow volume of the flow chamber includes means for immobilizing a free radical to receive microwave energy in said cavity and couple spin to nuclei flowing therethrough.

5. A probe according to claim 3 or 4, wherein the first coil spin locks nuclei as they traverse the microwave cavity.

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6. A probe according to claim 1, wherein the first and second coils each include a proton decoupling winding operable to suppress splitting during NMR detection through the coils.

7. A probe according to claim 1, wherein the first coil is tunable to spin-mark heteronuclear atoms, and wherein said flow chamber further comprises a reactor between said first and said second regions for initiating a reaction such that an analyte with a marked

heteronuclear atom undergoes a molecular change whereby heteronuclear detection at said second coil detects new molecular bonds of the marked atoms.

5 8. A probe according to claim 2. wherein said flow recirculation means further comprises a fluid-driven pump.

9. A probe according to claim 1. wherein said first and second flow volumes of said flow chamber have different cross-sectional areas.

10 10. A probe according to claim 9. wherein said cross-sectional areas differ by a factor greater than ten.

11. A probe according to claim 1. wherein said first and second flow volumes of said flow chamber define different flow axes.

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12. A probe according to claim 3. wherein said first coil and said microwave cavity are effective to apply mutually orthogonal fields to material flowing in said first region.

13. A probe according to claim 1. wherein said flow chamber further comprises delay means for varying flow time between said first and said second regions. without changing a flow rate entering said first region.

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14. A probe according to claim 13. wherein said delay means includes a telescoping conduit intermediate said first and said second flow volumes.

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15. A probe according to claim 13. wherein said delay means includes chromatograph column packing material held in said chamber upstream of said second flow volume.

16. The probe of claim 1. wherein said first coil and said second coil are tuned to the same nuclear resonant frequency.

30

17. The probe of claim 1. wherein said first and said second coil are tuned to different nuclear resonant frequencies.

35

18. The probe of claim 1. wherein at least one of said first coil and said second coil are selected from the group consisting of a Helmholtz coil. a Golay coil. a saddle coil. and a toroidal coil.



19. A probe for holding a sample of material in a test region of high magnetic field and uniformity in an NMR apparatus, such probe comprising:

5 a housing for positioning a sample-holding body in a test region and a body secured to said housing, said body having a flow chamber with an opening for introducing fluid to flow along a flow path through said test region, the flow chamber comprising in flow sequence a first flow volume disposed in a first region and a second flow volume disposed in a second region and connected immediately downstream of the first flow volume, so that flow through said first and second regions experiences the high field of said test region:

10 a first coil disposed in said first region and tunable to a first nuclear resonant frequency:

a second coil disposed in said second region and tunable to a second nuclear resonant frequency:

a microwave source disposed about said first coil:

15 a source of unpaired electrons disposed within said first coil:

whereby excitation of said unpaired electrons by said microwave source in combination with excitation of said first coil when a sample flows through said flow chamber enhances nuclear resonance detected by said second coil.

20 20. A method of performing an NMR measurement on a sample, such method comprising the steps of

placing the sample in a flow path defined within to a probe having a flow chamber with first and second RF coils disposed thereabout at successive upstream and downstream positions, respectively, along the flow path:

25 placing the probe in a magnetic field:

exciting the sample at one of said upstream and downstream positions: and

measuring a nuclear magnetic resonance spectrum at the other of said upstream and downstream positions.

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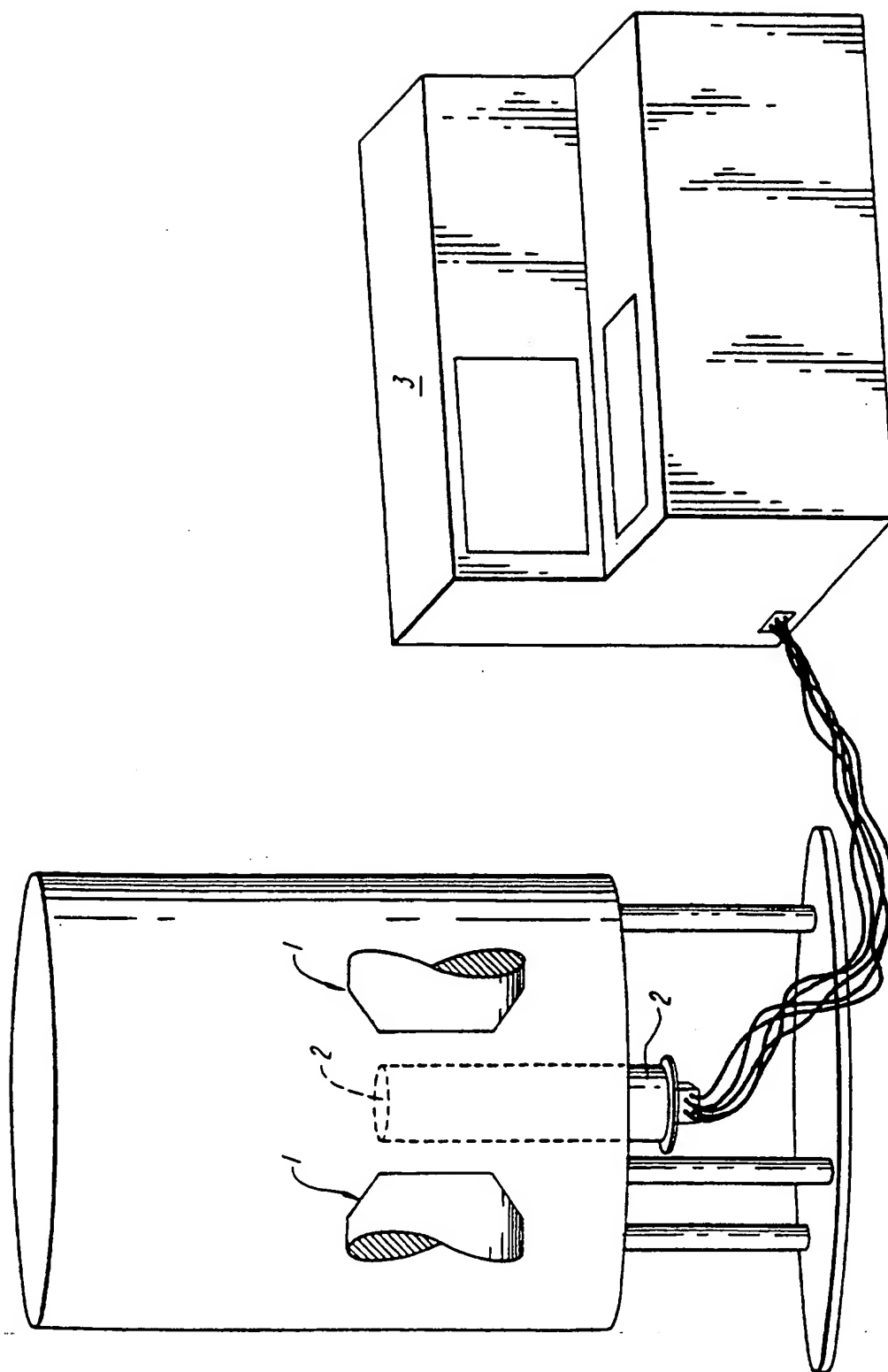
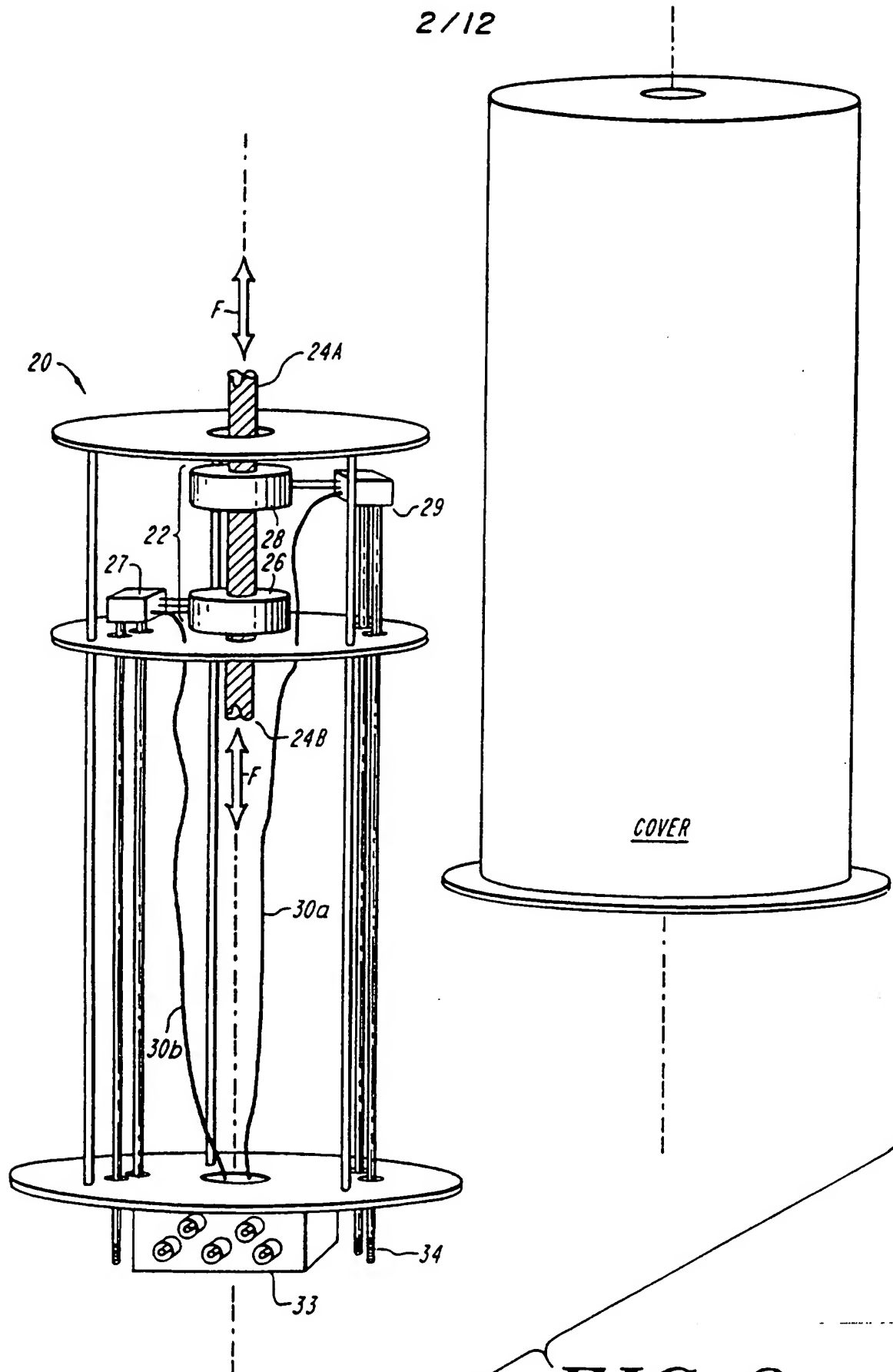
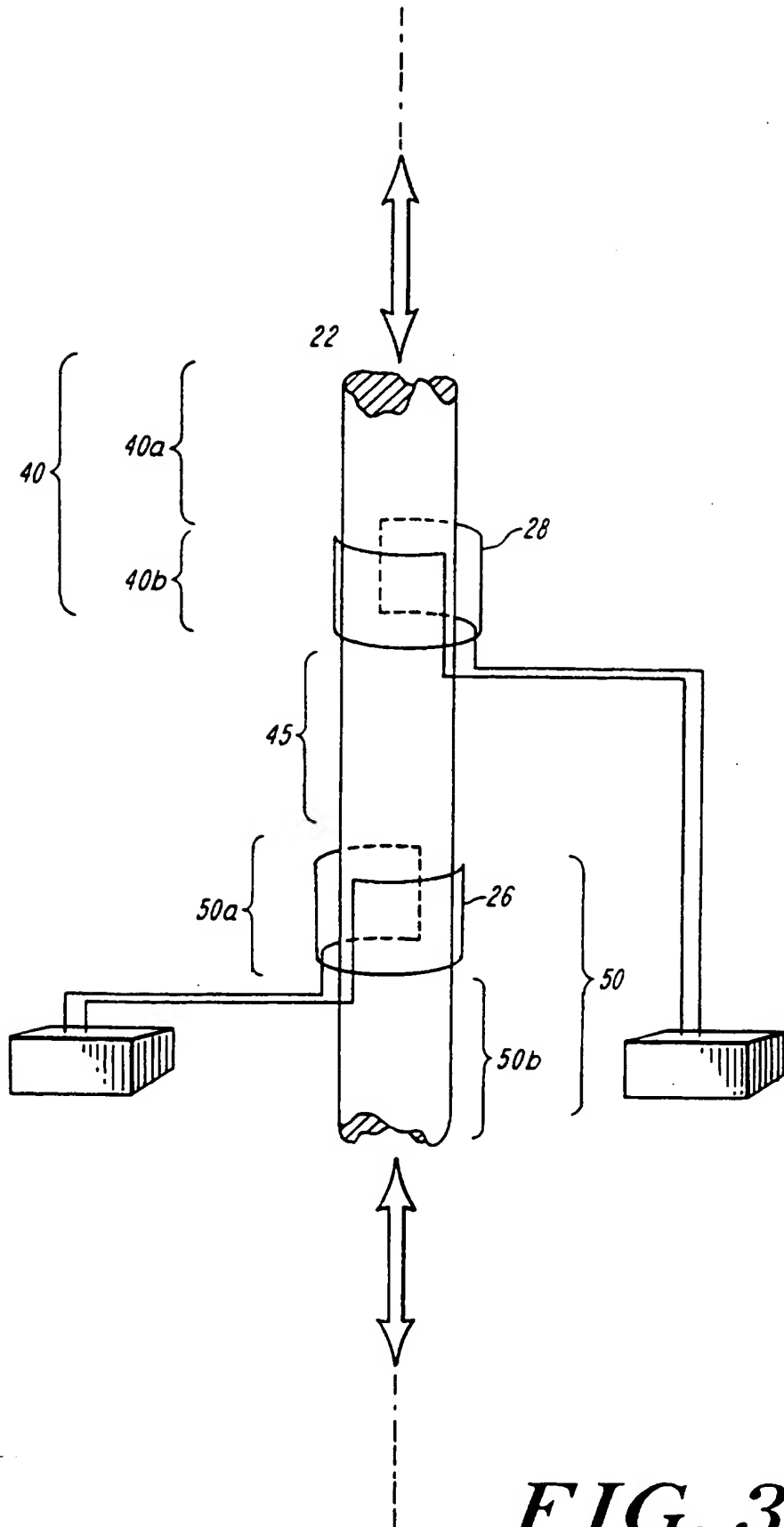


FIG. 1

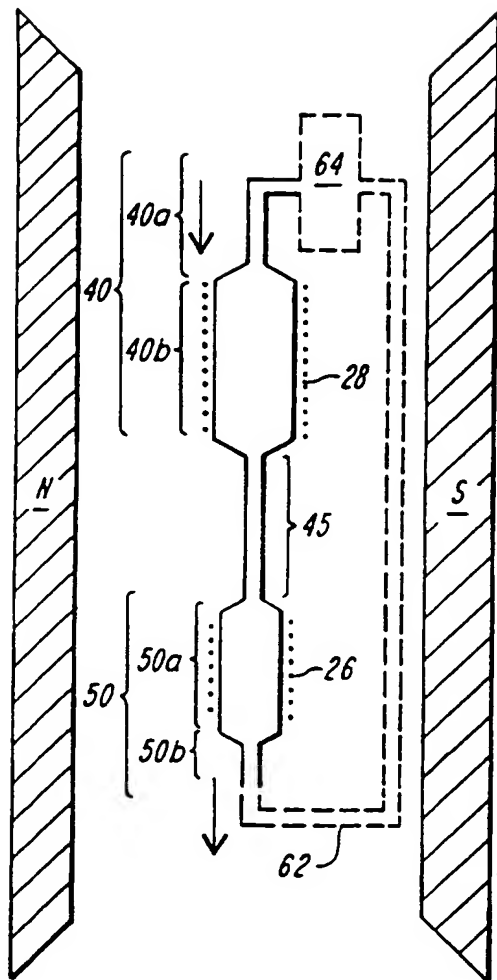
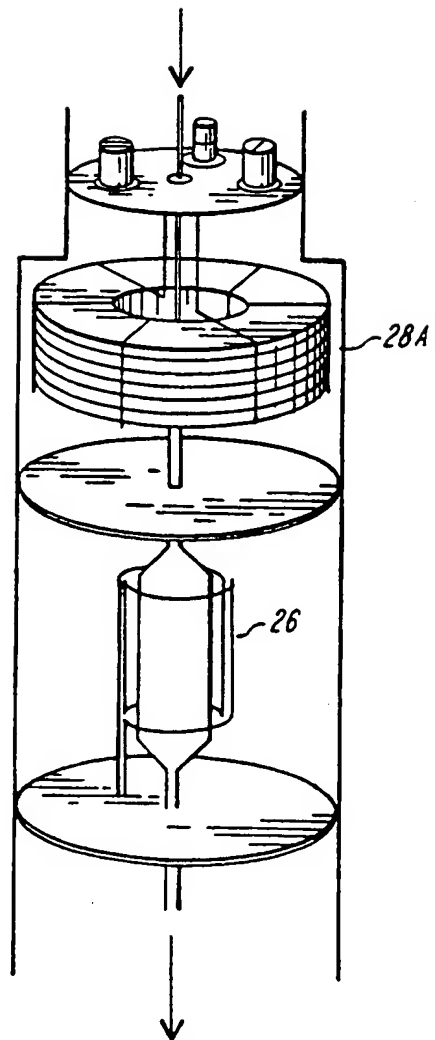
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**FIG. 2**

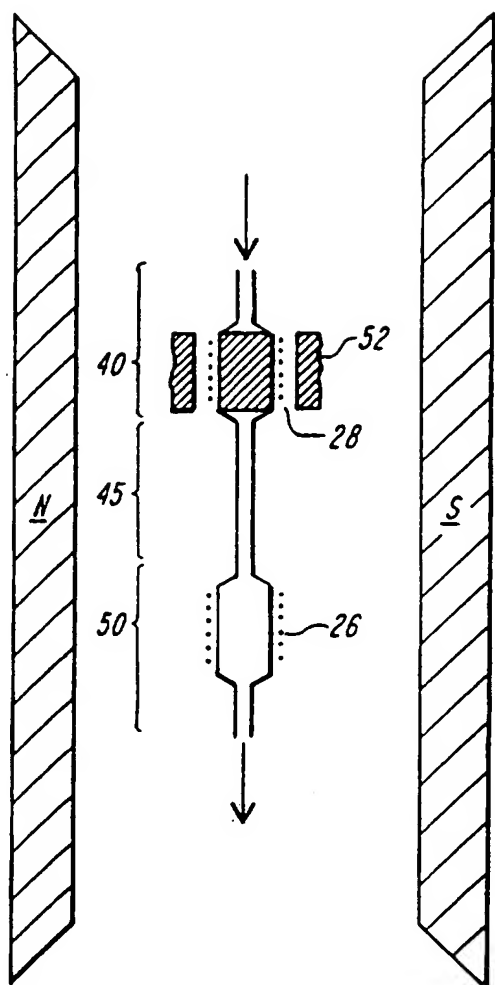
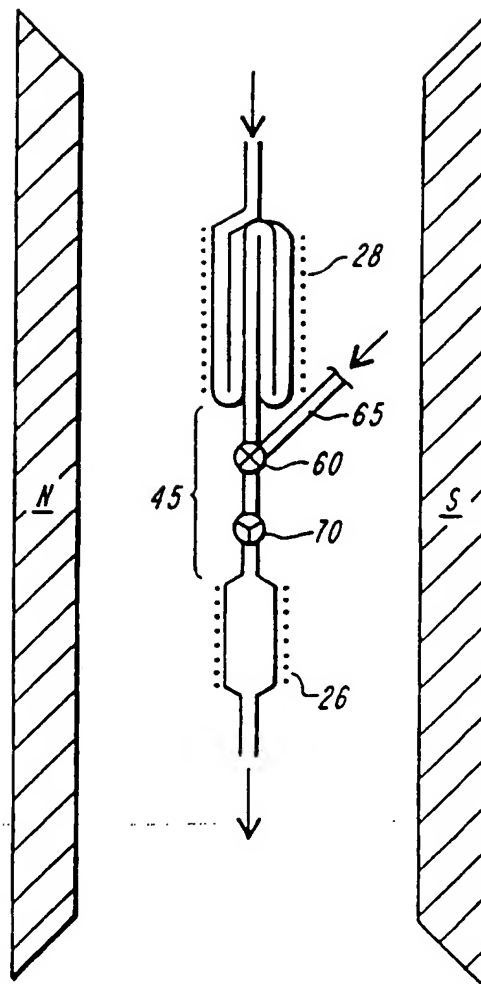
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**FIG. 3**

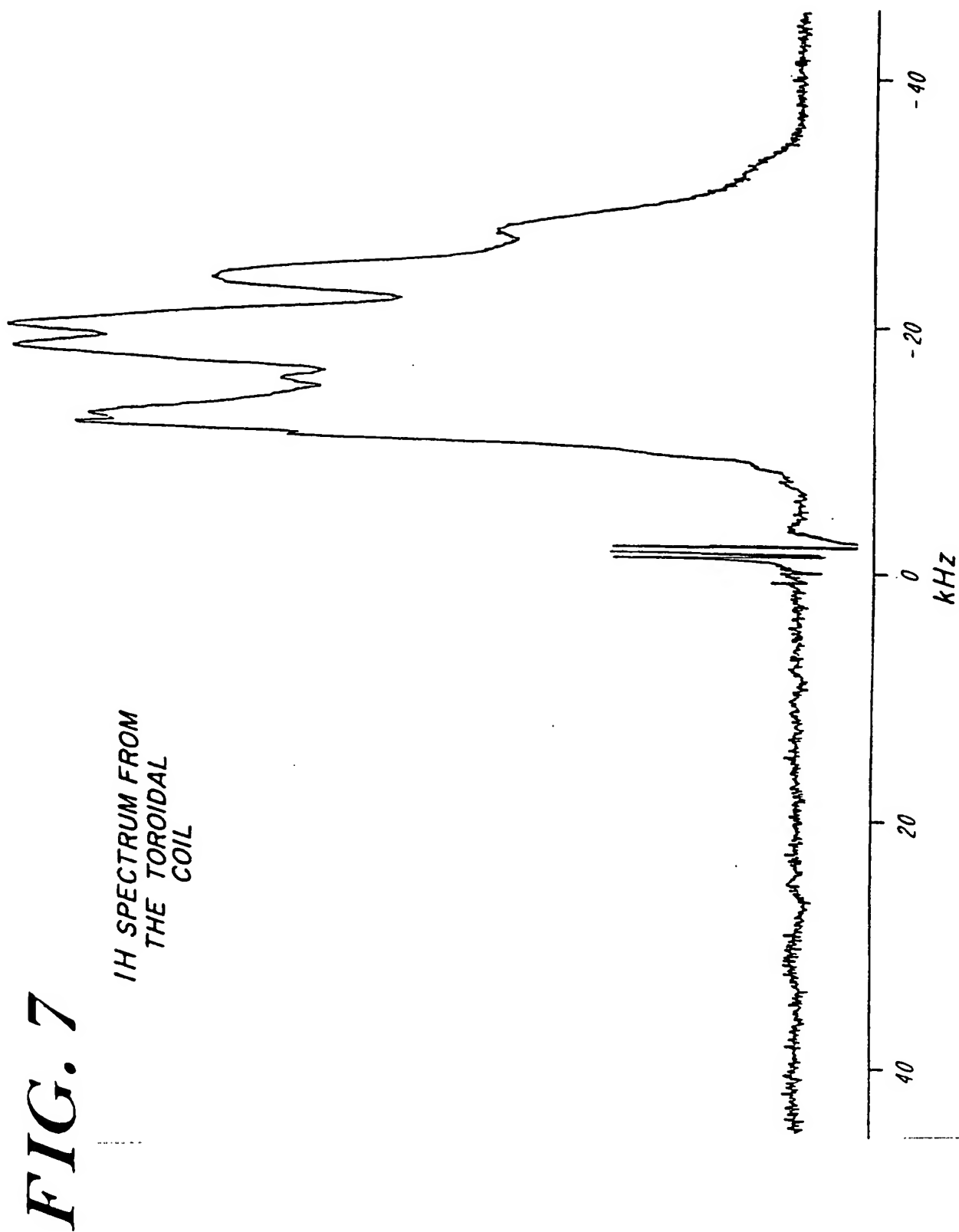
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**FIG. 4****FIG. 4A**

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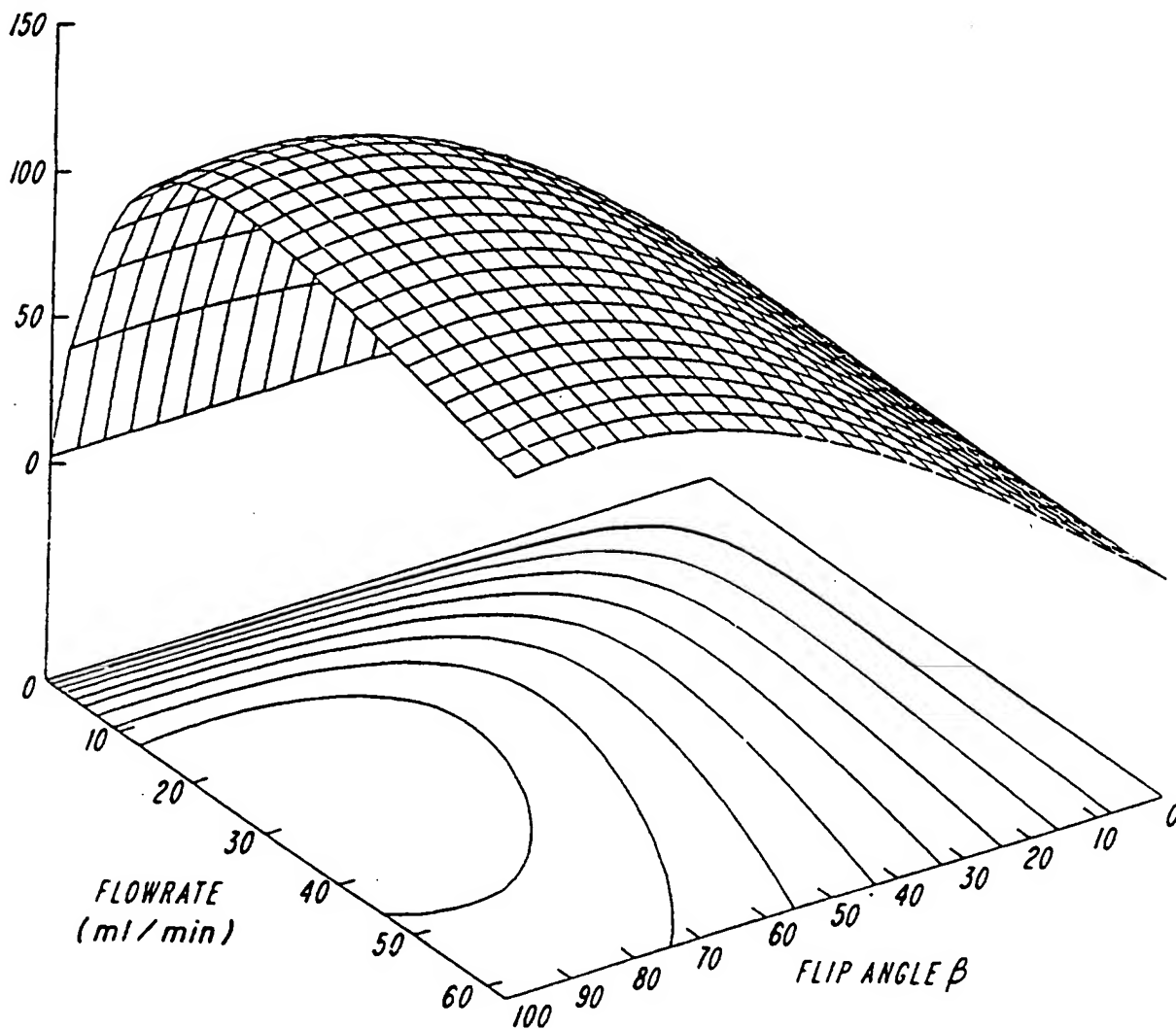
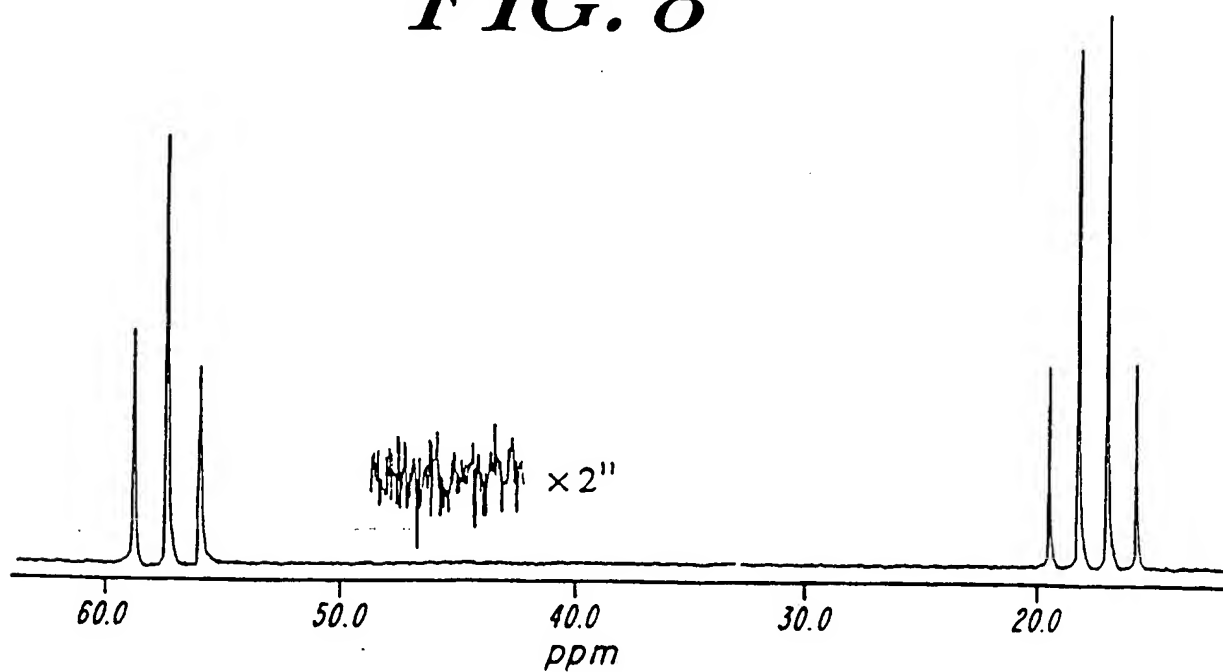
*FIG. 5**FIG. 6*

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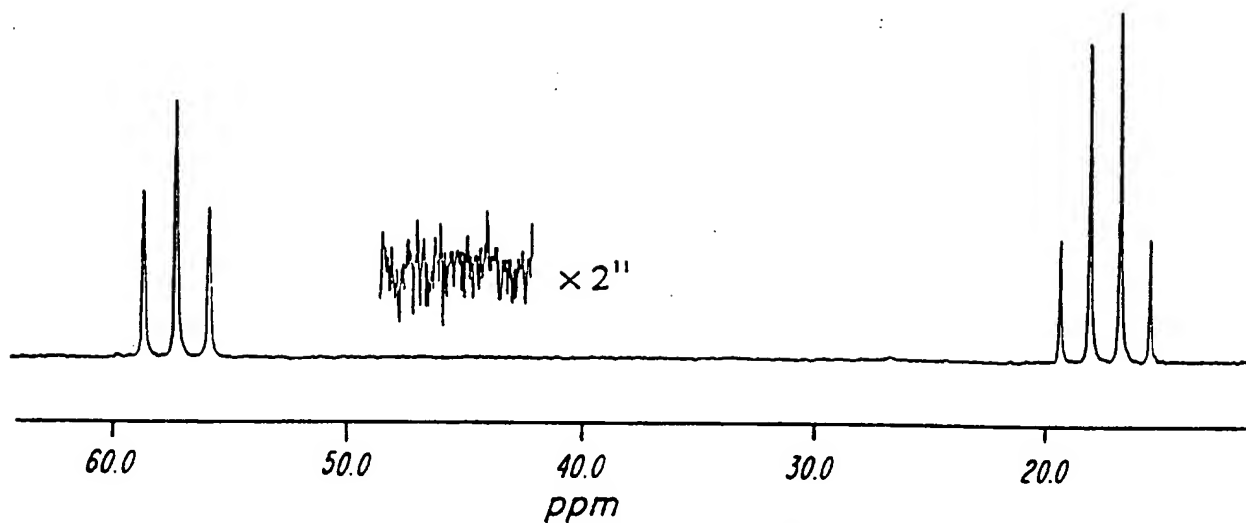
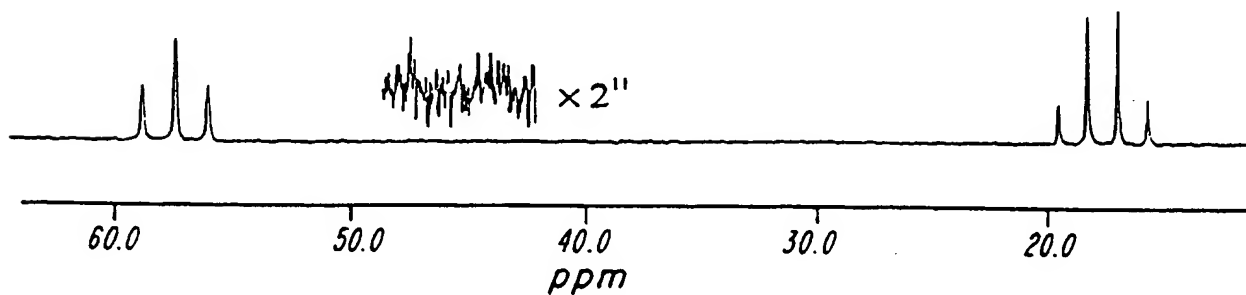
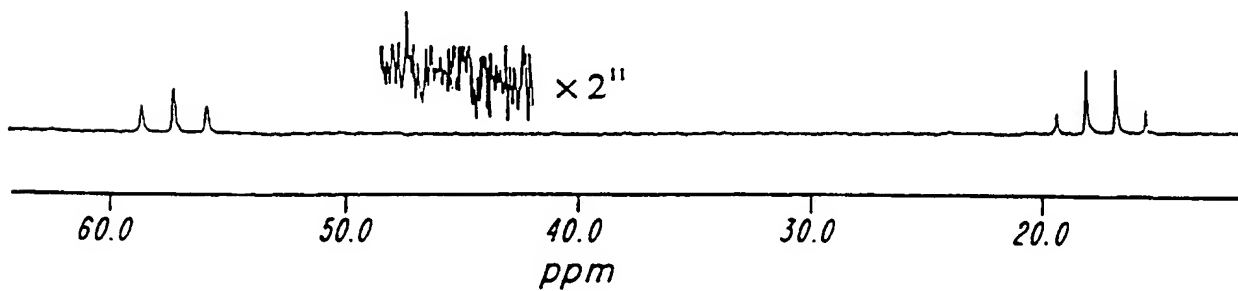
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SENSITIVITY

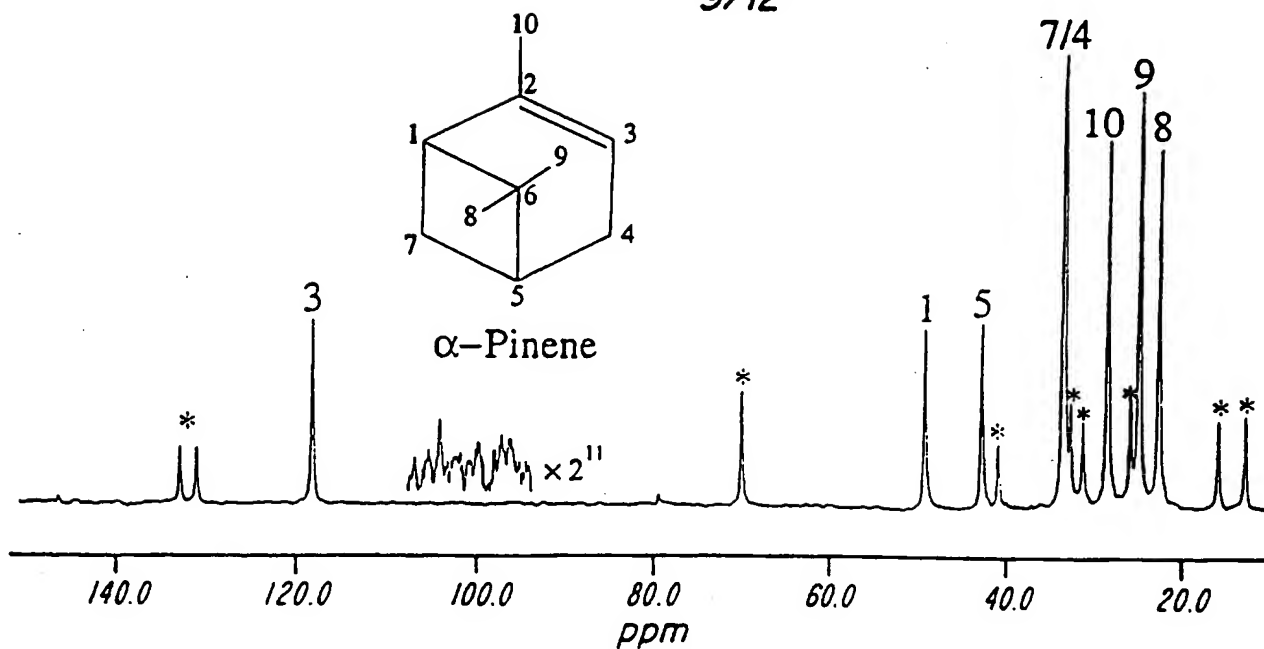
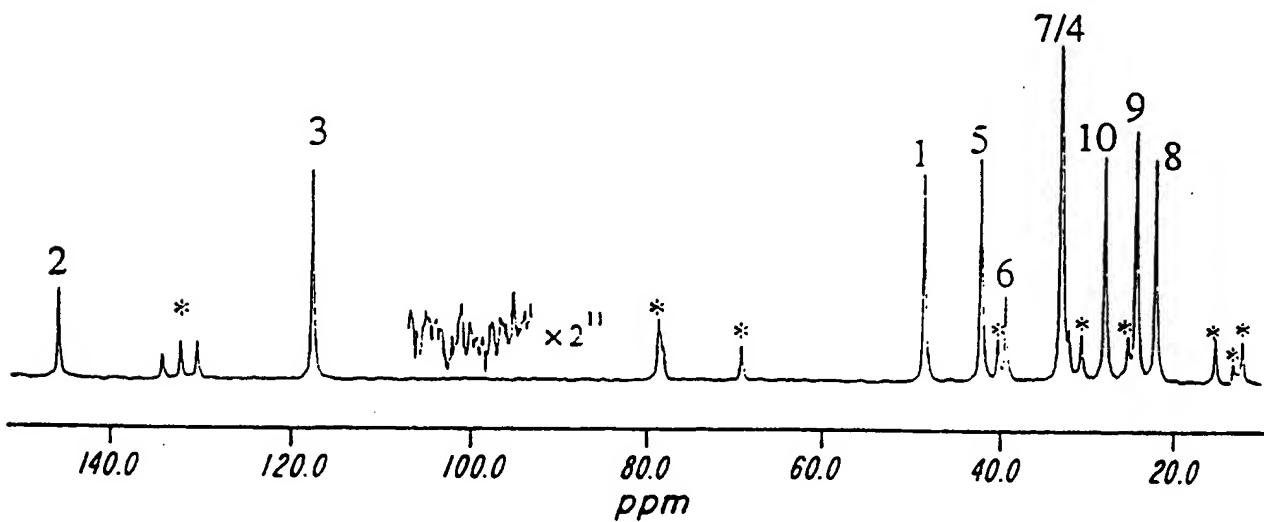
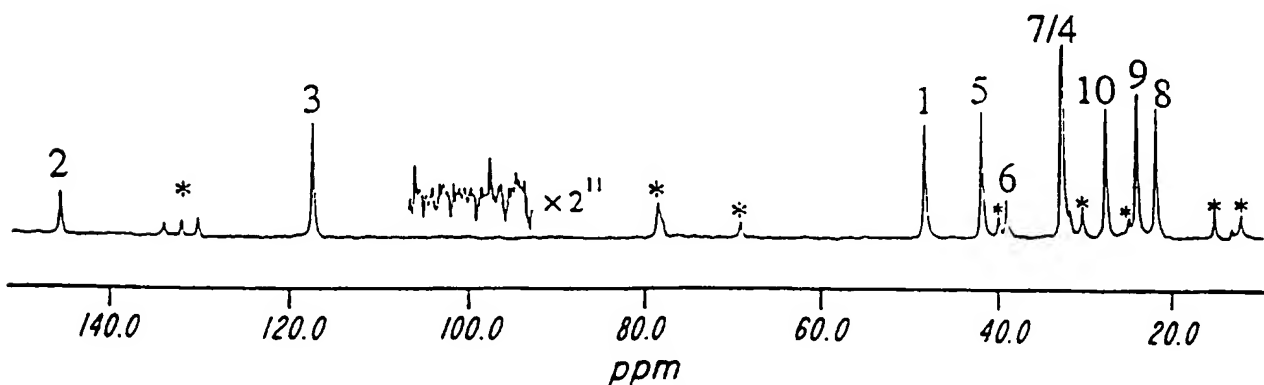
**FIG. 8****FIG. 9**



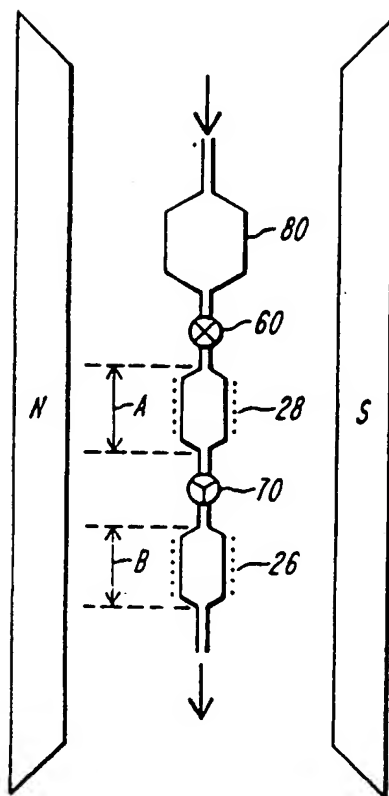
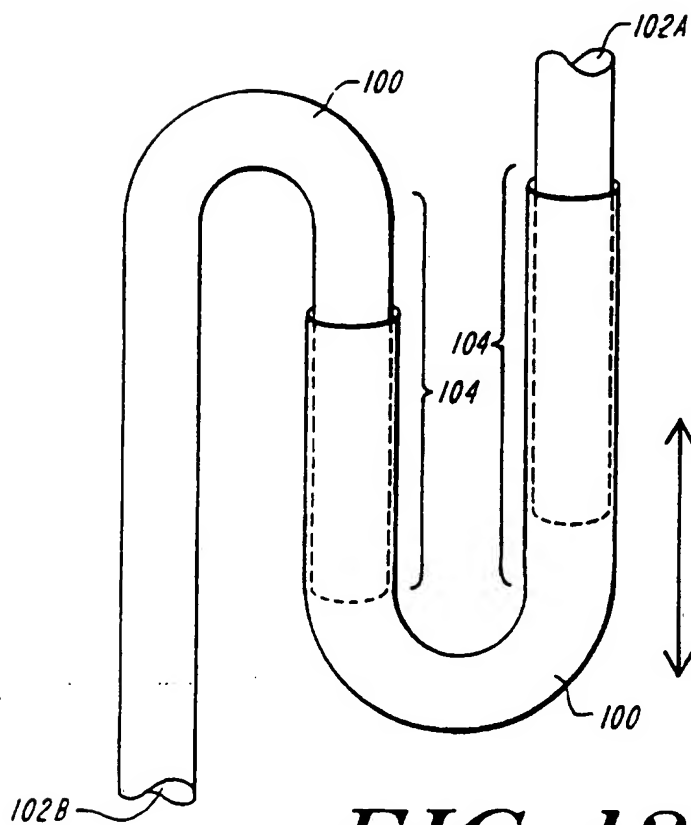
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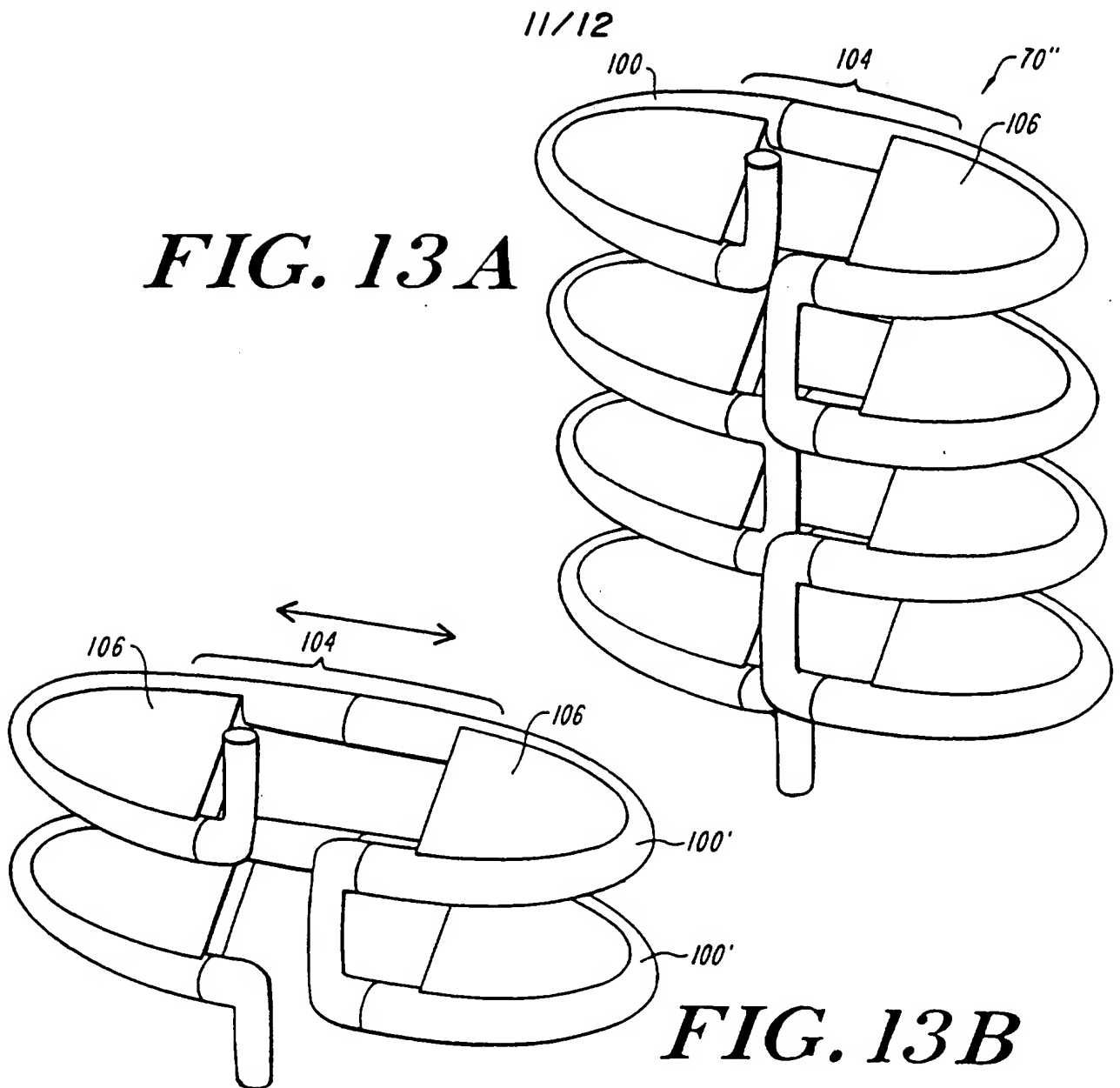
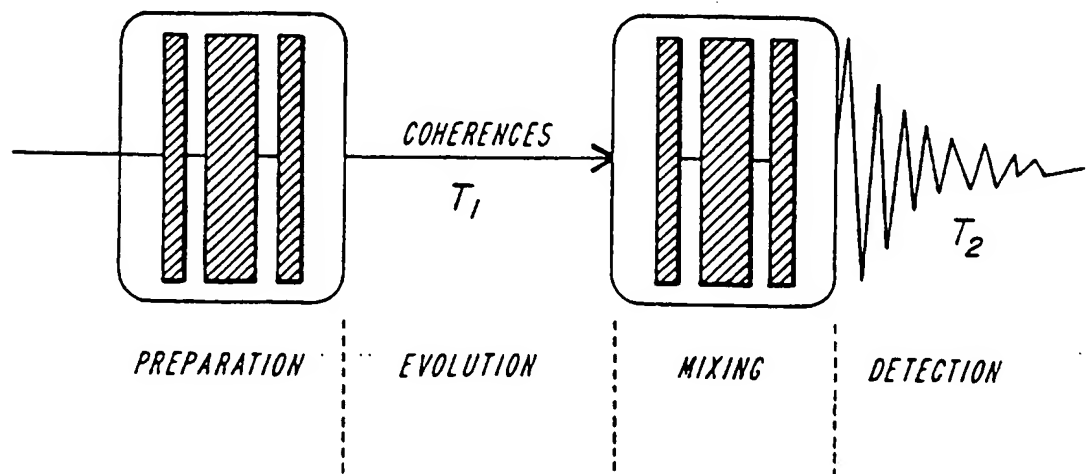
**FIG. 9C****FIG. 9B****FIG. 9A**

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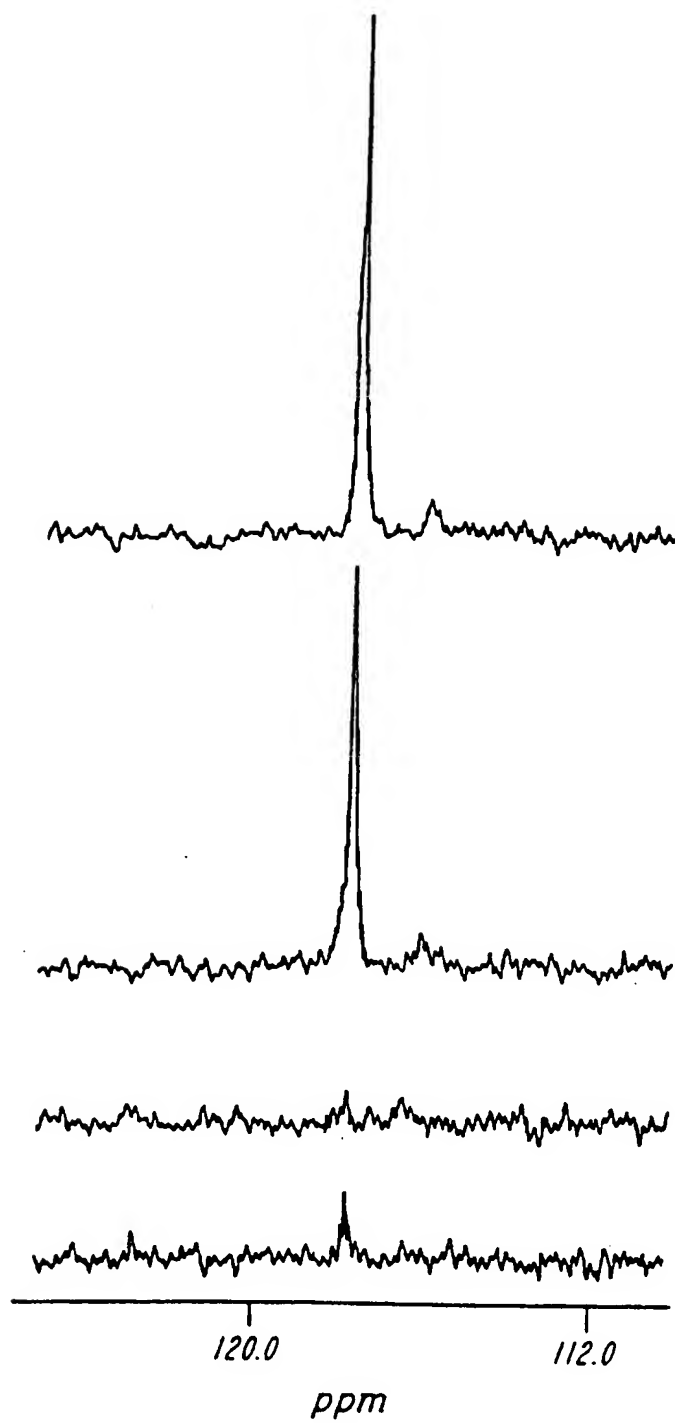
**FIG. 10C****FIG. 10B****FIG. 10A**

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**FIG. 11****FIG. 12**

**FIG. 13A****FIG. 13B****FIG. 14**

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***FIG. 15***

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : G01V 3/20, 3/28

US CL : 324/300, 303, 307, 309, 310, 311, 312, 313, 314, 318, 322

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 324/300, 303, 307, 309, 310, 311, 312, 313, 314, 318, 322

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,629,987 (KING ET AL) 16 December 1986	1-20
A	US, A, 4,638,251 (KING) 20 January 1987	1-20
A	US, A, 4,531,093 (ROLLWITZ ET AL) 23 July 1985	1-20
A	US, A, 5,352,979 (CONTURO) 04 October 1994	2, 6-15

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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*O* document referring to an oral disclosure, use, exhibition or other means	
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Date of the actual completion of the international search

07 FEBRUARY 1996

Date of mailing of the international search report

07 MAR 1996

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